MELATONIN AND THE CONTROL OF SEASONAL BREEDING IN THE SOAY RAM

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CONTENTS

DECLARATION

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author.

No part of this work has already been accepted in substance for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

O.F.X. ALMEIDA

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ABSTRACT

The studies described in this thesis were undertaken to investigate the role of melatonin (MEL), a secretion of the pineal gland, in the photoperiodic regulation of testicular activity in rams of the Soay breed. For this, rams were exposed to a variety of artificial light-dark (LD) cycles and their reproductive responses and temporal patterns of MEL in the blood were monitored.

Exposure of intact rams to short daylengths induced testicular development, whereas exposure to long daylengths resulted in testicular regression. While MEL was secreted maximally during periods of darkness in intact rams kept under short and long daylenghts, temporal differences, related to the duration of light and darkness, were observed in the profiles obtained under each photoperiod. There were no differences in the absolute amounts of MEL secreted.

Rams held under constant light or constant darkness for up to 10d maintained a 24h rhythm in their blood levels of MEL, suggesting that this rhythm was an endogenous circadian rhythm, with the LD cycle simply serving to entrain it.

Evidence that the blood MEL rhythm is a circadian one was obtained in a study in which rams were exposed to a photoperiod consisting of 8hL:40hD (48h cycle) or one of 8hL:28hD (36h cycle). In the 48h cycle, the 8h light period occurred at the same circadian time in each cycle, whereas in the 36h cycle it occurred at alternating circadian times. This protocol would test whether rams measure daylength by a circadian mechanism or by counting up the hours of light and darkness in each cycle. A consistent 24h MEL rhythm was found in the rams exposed to the 48h cycle, but not in those exposed to the 36h cycle, i.e. entrainment of the 24h MEL rhythm apparently depends on a LD cue occurring at 24h intervals or multiples thereof.

In the above experiment, the testes of the rams under the 48h cycle became fully developed as though the rams were exposed to ordinary short daylengths whereas those of the rams under the 36h cycle became only partially developed. Thus a circadian mechanism also appears to underly the photoperiodic regulation of reproduction, and there may be a correlation between blood patterns of MEL, photoperiod and reproductive status.

Correlations between blood patterns of MEL and reproductive state were also obtained in an experiment in which rams were exposed to a prolonged period (96 weeks) of either long or short daylengths. The animals eventually became refractory to the normal reproductive effects of each of these photoperiods, showing gonadal involution and recrudescence despite the daylengths they were exposed to. The 24h pattern in the blood levels of MEL were found to become disrupted concomitantly with the onset of photo-refractoriness.

In an attempt to find the site of action of MEL, rams were actively immunized against the hormone. Despite the achievement of reasonable antibody titres, the photoperiodic responses of the rams were not disturbed, suggesting that MEL might act within the CNS to which the antibodies would have no access.

A central site of action is presumed in a model for the way in which MEL might be involved in the photoperiodic regulation of reproduction in the ram. It is proposed that its principal role is to relay information about daylength to the hypothalamic-pituitary-gonad axis. The patterns of MEL secretion closely reflect the LD cycle through a circadian mechanism. In addition, there is a circadian rhythm in brain sensitivity to MEL. Interpretation of the photoperiod, and thus gonadal activation or involution, is then based upon the phase relationships of the rhythms in MEL release and the sensitivity of the brain to MEL.

GLOSSARY

SYMBOLS

CSF:	Cerebrospinal fluid
BSA:	Bovine serum albumin
FSH:	Follicle stimulating hormone
GnRH:	Gonadotrophin-releasing hormone
LD:	Light-dark cycle. A light-dark cycle is composed of light time (L) and dark time (D); the term 'photoperiod' is used synonymously with LD cycle
n_1L:n_2D:	n_1 hours light : n_2 hours darkness
LL;DD:	Continuous illumination; continuous darkness
LH:	Luteinizing hormone
MEL:	Melatonin
PAGE:	Polyacrylamide gel electrophoresis
PINX:	Pinealectomy
PRL:	Prolactin
RHT:	Retinohypothalamic tract
RIA:	Radioimmunoassay
SCG:	Superior cervical ganglion (ganglia)
SCGx:	Superior cervical ganglionectomy

TERMS*

Amplitude:	Difference between maximum (or minimum) and mean value in an oscillation
Daytime:	Period of illumination
Entraining agent:	see zeitgeber
Entrainment:	Synchronization of a self-sustaining oscillation by a forcing oscillation (zeitgeber). During entrainment, the frequencies of the two oscillations are the same or integral multiples.
Free run:	State of a non-entrained rhythm, either in constant conditions or after loss of entrainment by a zeitgeber that is still present but too weak.
Long days:	Long daily photoperiods
Night-time:	Period of darkness

Phase:	Instantaneous state of an oscillation within a period, represented by the value of the variable and all its time derivatives.
Phase angle:	Value of the abscissa corresponding to a phase of the oscillation.
Phase shift:	Single displacement of an oscillation along the time axis; may occur instantaneously or after several transients.
Phase relation:	General term for the time-relation between two coupled oscillations.
Period:	Time after which a definite phase of an oscillation recurs.
Short days:	Short daily photoperiods
Zeitgeber:	The forcing (external) oscillation that entrains a biological (self-sustaining) oscillation.

*Taken from: Handbook of Neurobiology, Vol.4: "Biological Rhythms". J. Aschoff, Ed. p.547. Plenum Press, New York, 1981.

PUBLICATIONS RELATED TO THE STUDIES DESCRIBED

ALMEIDA, O.F.X. & LINCOLN, G.A. (1980): Circadian rhythms in melatonin and prolactin secretion in the Soay ram. Poster No. 85: 161st Meeting of the Society for Endocrinology, London. November, 1980. (Abstract).

LINCOLN, G.A. & ALMEIDA, O.F.X. (1981): Photoperiodism and the nature of a ram's inhibitions. Paper No. 90: Society for the Study of Fertility, Annual Meeting, Edinburgh 1981. (Abstract).

LINCOLN, G.A. & ALMEIDA, O.F.X. (1981): Melatonin and the seasonal photoperiodic response in sheep. In: "Photoperiodism and Reproduction in Vertebrates". INRA Colloque International 6, 231-251.

LINCOLN, G.A., ALMEIDA, O.F.X. & ARENDT, J. (1981): Role of melatonin and circadian rhythms in seasonal reproduction in rams. Journal of Reproduction & Fertility Supplement 30, 23-31.

LINCOLN, G.A., ALMEIDA, O.F.X., KLANDORF, H. & CUNNINGHAM, R.A. (1982): Daily rhythms in the blood levels of melatonin, prolactin, LH, FSH, testosterone, T_3, T_4 and cortisol in rams under artificial photoperiods, and the effects of cranial sympathectomy. Journal of Endocrinology 92, 237-250.

RENFREE, M.B., LINCOLN, D.W., ALMEIDA, O.F.X. & SHORT, R.V. (1981): Abolition of seasonal embryonic diapause in the tammar wallaby by sympathetic denervation of the pineal gland. Nature 293, 138-139.

SHARP, P.J., ALMEIDA, O.F.X., KLANDORF, H. & LEA, R.W. (1981): The effect of pinealectomy on daily rhythms in plasma concentrations of melatonin and on the onset of puberty in the domestic hen. Paper No.64: 164th Meeting of the Society for Endocrinology, London. November, 1981. (Abstract).

IN PRESS

ALMEIDA, O.F.X. & LINCOLN, G.A. Central mechanisms in the control of seasonal breeding. Proc. Symp. "Physiological Problems of Seasonal Breeding", 3rd Int. Theriological Congress, Helsinki. August, 1982. Acta Fennica Zoologica.

ALMEIDA, O.F.X. & LINCOLN, G.A. Photoperiodic regulation of reproductive activity in the ram: evidence for the involvement of circadian rhythms in melatonin and prolactin secretion. Biology of Reproduction.

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 THE MAMMALIAN PINEAL

1.1.1 Historical Perspective

The Greek anatomist Herophilus and his followers (ca.300 B.C.) propounded the idea that the ventricles of the mammalian brain were the "seat of the mind". This school also suggested that the pine cone-like body in the third ventricle was a sphincter, serving to regulate the flow of thought (Kitay & Altschule, 1954). The ancient Greeks called this body Konarion (Singer, 1956) and, in classical anatomy it is known as the epiphysis cerebri or conarium. Today it is more commonly called the pineal as suggested by Thomas Willis (1621-75).

Another Greek, Galenus (132-200 A.D.), in his notes on the dissection of the brain, described the location of the pineal in relation to other brain structures. Galen refuted the concept of the pineal as a sphincter, suggesting instead that it might be a gland, similar to the lymph glands (Singer, 1956; Kitay & Altschule, 1954; Kappers, 1981).

Rene Descartes (1596-1650 A.D.), the philosopher and physician, designated the pineal as the "seat of the soul", since it is the only unpaired organ in the brain (Kappers, 1979). Descartes also proposed that the pineal received information from the eyes and other sense organs (Fig. 1.1).

Credit for recognition of the pineal as an endocrine gland should be given to Thomas Gibson. Reviving Galen's analogies to describe the area surrounding the pineal, Gibson, in his "Epitome of Anatomy", published in 1763, wrote:-

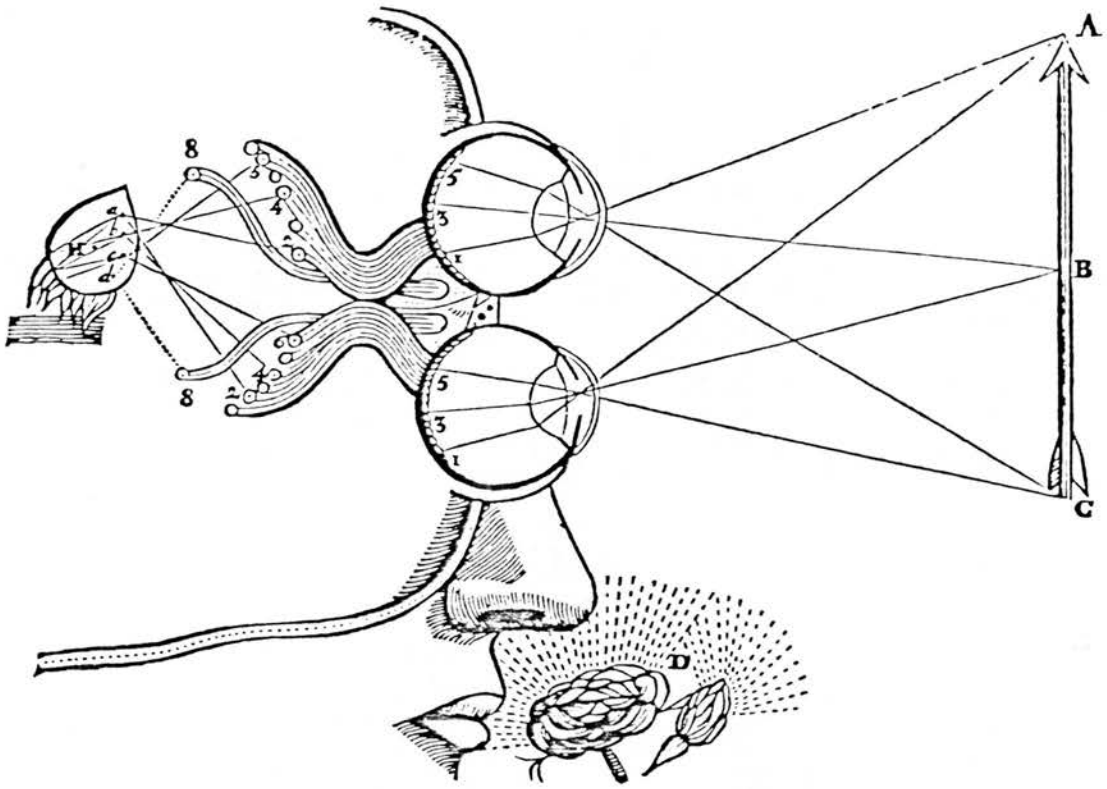


Fig. 1.1 Drawing from Rene Descartes' "Tractus de Homine", in which he depicts the pineal as a single, cone-shaped body serving as the centre of all sensory input into the brain. Descartes proposed that the pineal is the "seat of the soul". (Photograph by courtesy of the Library of the Royal Society of Medicine, London).

The first is Glandula pinealis, or Penis; because it representeth the Pine-nut, or a Man's Yard. It is seated in the beginning of that Pipe by which the third and fourth Ventricles are united. Its basis is downwards, and its apex or end looks upwards. It is of a substance harder than the Brain, of a pale colour, and covered with a thin Membrane. This Gland des Cartes thinks to be the primary seat of the Soul, and that all animal operators draw their origine from it. But Bartholin has sufficiently confuted that opinion; for it seems to be but of the same use as other Glands, and particularly the Glandula pituitaria placed near it, viz. to separate the Lympha from the Arterial blood; which Lympha is resorbed by the Veins (or it may be by Vasa lymphatica) as was shown from Dr. Lower. Near to this on both sides of this third Ventricle four round bodies appear. The two upper are lesser, and are called Testes: the two greater are lower, and are called Nates. The chink betwixt the Nates is called Anus.

Gibson's writings led to the idea that the pineal was the "penis of the brain". Despite the connotations of this term, the link with reproduction was not made until 1898 when the French clinician Heubner described precocious sexual development in a four-year old boy who was found to have a pineal tumour at post mortem.

A more complete history of the discovery and early embryological, anatomical and physiological studies of the pineal is provided by Rolleston (1936). In 1954, Kitay & Altschule reviewed the literature on comparative and clinical aspects of pineal function. It is their book, "The Pineal Gland" which triggered much research into pineal physiology.

The first pineal hormone to be isolated was the methoxyindole, N-acetyl-5-methoxytryptamine (melatonin, MW 232). It was extracted and characterised from bovine pineals by Lerner and his colleagues (Lerner, Case, Takahashi, Lee & Mori, 1958; Lerner, Case & Heinzelman, 1959) who were seeking the pineal substance that caused the blanching of frog and tadpole skins (McCord & Allen, 1917). Lerner called the highly potent pineal skin-lightening agent "melatonin" since it antagonised the effects of melanocyte stimulating hormone from the pars intermedia of the pituitary. The synthesis of melatonin was described by

Szmuszkovicz, Anthony & Heinzelman (1960). Of all the pineal compounds identified, melatonin has received the greatest attention (Cardinali, 1981; Birau & Schloot, 1981). There are now several chemical and immunological methods for the assay of melatonin in a variety of body fluids and tissues (Quay, 1974; Lewy & McKay, 1978; Arendt, 1981), but the skin-lightening effects on frog and tadpole skins still remains the only biological assay for melatonin (Lynch, Ozaki & Wurtman, 1978).

1.1.2 Evolution

Distinct pineal bodies, occurring as dorsally-projecting structures on the diencephalon have been found in most vertebrates. The few exceptions are the hagfish (Myxine), the crocodiles, endentates and dugongs (Oksche, 1965), although there is evidence that diffuse regions of pineal tissue might exist along the diencephalic brain roof in some of these species (Wurtman, Axelrod & Kelly, 1968; Roth, Gern, Roth, Ralph & Jacabson, 1980; Harlow, Phillips & Ralph, 1981). Even the most primitive of living fishes, the jawless fishes, have pineal systems (Edinger, 1956), pointing to the age of the vertebrate pineal. During the course of evolution, the vertebrate pineal has shown transformations in structure, function and innervation. In the lower vertebrates, "pineal systems" consisting of a pineal organ and parapineal tissues have been described, whereas distinct pineal glands are found in birds and mammals (Wurtman et al, 1968; Collin, 1971; Kappers, 1971).

The pineal cell has transformed from being a sensory cell concerned with photoreception in the fishes, amphibians and lacertilian reptiles, to an endocrine cell in snakes, turtles, birds and mammals. There is good evidence that the avian pinealocyte has retained some of its photoreceptive properties and can respond directly to light

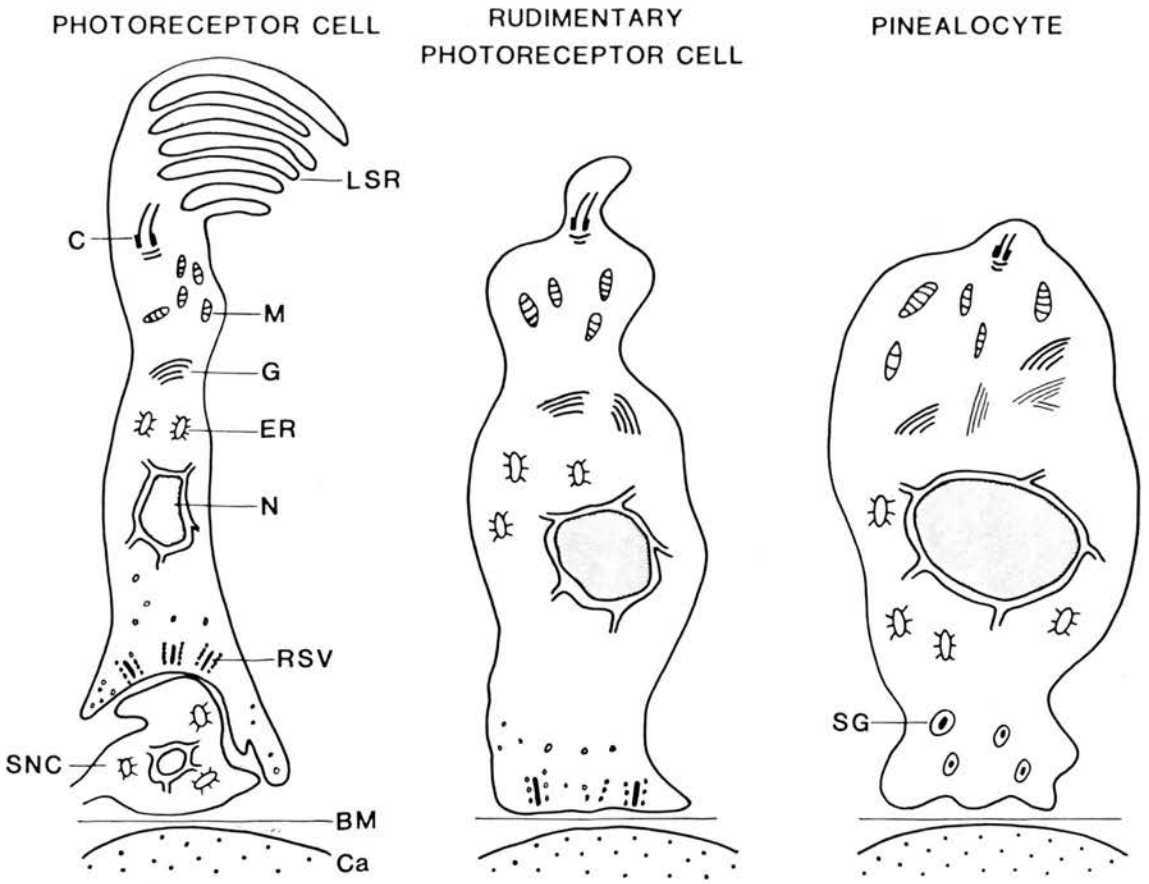


Fig. 1.2 Schematic representation of the transformation of the photosensory cells of primitive vertebrates into mammalian pinealocytes. Photoreceptor cells occur in the cyclostomes, fishes and amphibians, and to some extent in the chelonian and lacertilian reptiles. Rudimentary photoreceptor cells are found in the chelonians, lacertilians and birds; their existence has also been tentatively suggested in mammalian embryos. Pinealocytes are found in mammals. They are also found in snakes, and probably occur in small numbers in the chelonians, lacertilians and birds.

Abbreviations: b.M, basement membrane; C, centriole; ca, blood capillary; e.r., endoplasmic reticulum; G, Golgi apparatus; l.s.r. light-sensory region, m, mitochondrion; n, nucleus; s.g. secretory granule; r.s.v., synaptic ribbon with vesicles; s.n.c., sensory nerve cell. (drawn from: Collin, 1971; Hamaski & Elder, 1977).

(Deguchi, 1979, 1981), whereas the mammalian pineal cell has apparently lost all its photoreceptive functions. The evolution of the mammalian pinealocyte has been reviewed by Collin (1971) and Hamaski & Eder (1977), and their studies are summarised in Fig.1.2.

The neural connections of the pinealocyte with the rest of the brain have also changed during evolution. The pineal organs of the lower vertebrates send sensory nerve bundles to the brain, whereas in birds and mammals, the pineal gland only receives autonomic fibres from the brain. In the mammalian foetus, however, pineal embryology is seen to recapitulate phylogeny; nervous input from the pineal into the brain has been described (Kappers, 1965; Quay, 1974; Ueck, 1979; Lincoln, 1983).

The endocrine function of the mammalian pineal is regulated by the environmental light-dark cycle (Reiter, 1980; Cardinali, 1981). For this, it depends upon its autonomic innervation from the brain. The avian pineal also responds to changes in the external lighting but its responses appear to be independent of its innervation (Binkley, 1980; Menaker, Hudson & Takahashi, 1981); this presumably reflects the photoreceptive properties of the avian pineal cell.

1.1.3 Anatomical Aspects

The relative size of the pineal gland varies markedly within the mammalian class. In a review of the literature, Ralph (1975) concludes that larger pineals are found in mammals of high latitudes than in those of the tropical and equatorial regions. The relative sizes of the pineals of four mammals are shown in Fig. 1.3. The significance of such differences will be discussed in Section 1.4 of this chapter.

Mammalian pineals differ greatly in shape. In some species, the pineal is a single-structured organ whereas in others it may consist of

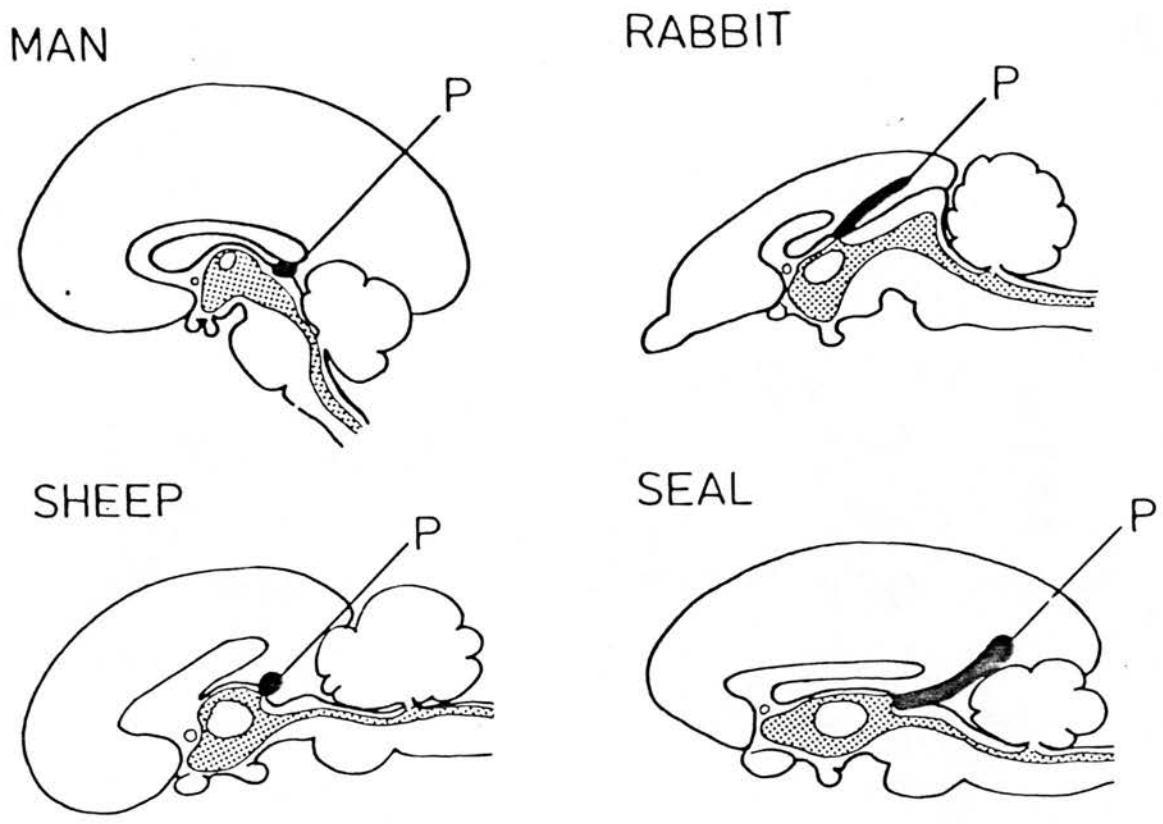


Fig. 1.3 Examples of variation in the size and shape of the pineal gland in four mammalian species. Note the relatively large pineal of the Weddell seal. This species is found in the cool temperature latitudes and shows a marked seasonal reproductive cycle (from: Lincoln, 1983).

a complex of several distinct lobes. There are five classes of pineal complexes; these are reviewed by Vollrath (1979).

The fine structure of the pinealocyte has been extensively reviewed by Wurtman et al (1968), Kappers (1971), Oksche (1971) and Quay (1974). The mammalian pineal cell consists of a large cell body and a synaptic pedicle which extends towards a perivascular space (Fig. 1.2). The synaptic pedicle has a basement membrane through which it makes contact with nerve fibres. Secretory granules are frequently observed within the synaptic pedicle, apparently migrating from the cell body towards the basement membrane. The cell body itself comprises a large nucleus, a Golgi apparatus and rough endoplasmic reticulum.

The mammalian pineal is a highly vascularised organ. The pineal parenchyme receives its blood supply from the posterior choroid arteries (Quay, 1974). The blood capillaries are separated from the pineal cells by a well-developed perivascular space into which the pinealocytes send cytoplasmic extensions (Reiter, Vaughan & Black, 1975). The mammalian pineal is surrounded by meninges, and the gland lies in close proximity to the 3rd ventricle which is filled with cerebrospinal fluid (CSF). There do not appear to be any structural links such as interstitial channels or canniculi between the pineal and the CSF (Reiter et al, 1975).

Our knowledge of the neural connections of the pineal gland stem mainly from studies in rodents. The mammalian pineal does not send sensory nerve fibres into the central nervous systen (CNS). The only neural link between the pineal and the brain is through the autonomic nervous system. The major innervation comes from the sympathetic

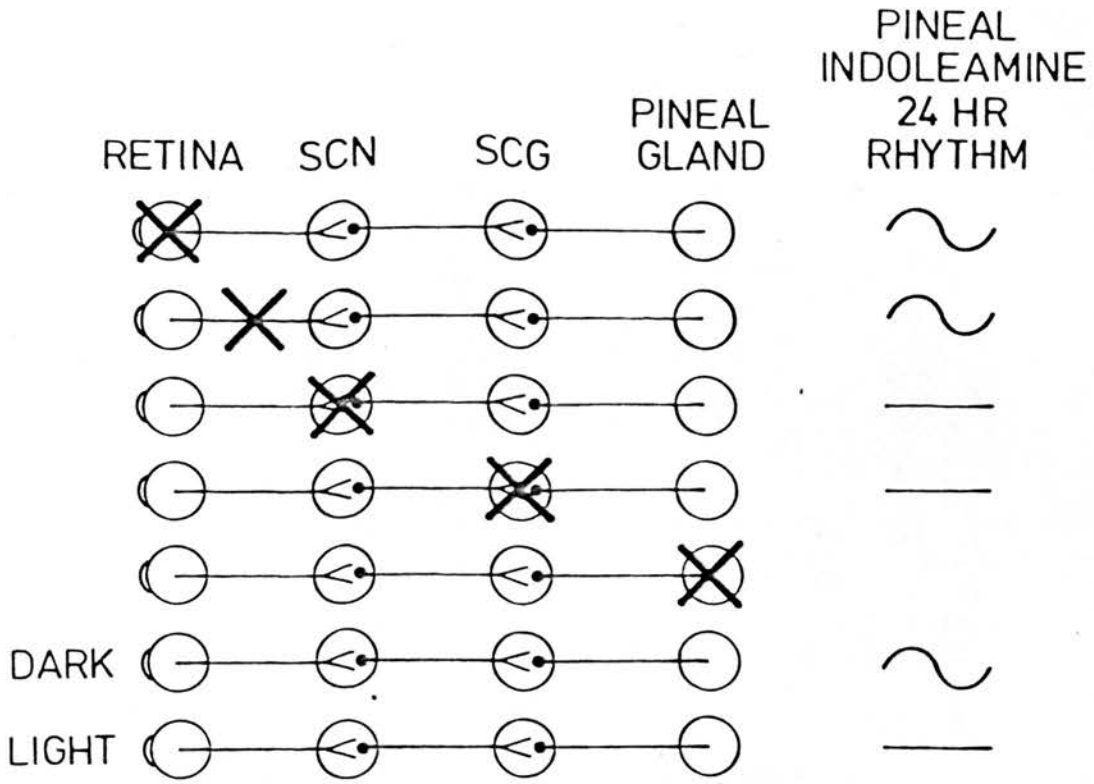


Fig.1.4 The neural structures involved in transmitting photic information to the mammalian pineal. The involvement of these structures was shown by studying changes in the rhythmical production of pineal indoleamines in response to the various lesions (X) shown. The initial studies were performed in the laboratory rat, but have since been extended to, and confirmed in, a number of other mammalian species (see Moore, 1979). The direct link between the retina and the SCN- the retinohypothalamic tract - has been shown to exist in the sheep (Legan & Winans, 1981). The neural pathway between the SCN and SCG has not yet been identified in any species (Drawing from: Lincoln, 1983).

nerves originating in the superior cervical ganglia. Some of these fibres enter along with the pineal blood vessels, while others reach the caudal pole of the pineal by way of the nervi conarii. The axons branch extensively and terminate in the perivascular space, in close proximity to the pinealocytes (Kappers, 1965; Quay, 1974; Moore, 1978; Ueck, 1979). Recently, a more direct CNS sensory input to the pineal has been described; fibres linking the habenula with the pineal have been identified in several rodents (Ueck, 1979; Semm, Schneider & Vollrath, 1981; Dafny, 1980).

The sympathetic nerve supply to the pineal has been found to be essential for transmitting information about the environmental light-dark (LD) cycle to the pineal. The neural route by which photic information reaches the gland has been mapped by lesion studies accompanied by studies of pineal chemistry (Fig.1.4.). Such studies indicated that the primary optic nerves are not involved, since blinding does not disturb pineal function. Lesions placed between the suprachiasmatic nuclei (SCN) and the pineal did, however, alter pineal chemistry. This led to the discovery of the retino-hypothalamic tract (RHT) which links the eyes with the SCN, independently of the primary visual tracts. The nerve impulse travels from the SCN to the superior cervical ganglia (SCG) and thence to the pineal gland (Moore, 1978, 1979). The proposed neural pathway is shown in Fig.1.5. The pathway was first described for the rat, but there is now evidence for its existence in other mammals including the hamster (Stetson & Watson-Whitmyre, 1976) and sheep (Legan & Winans, 1981).

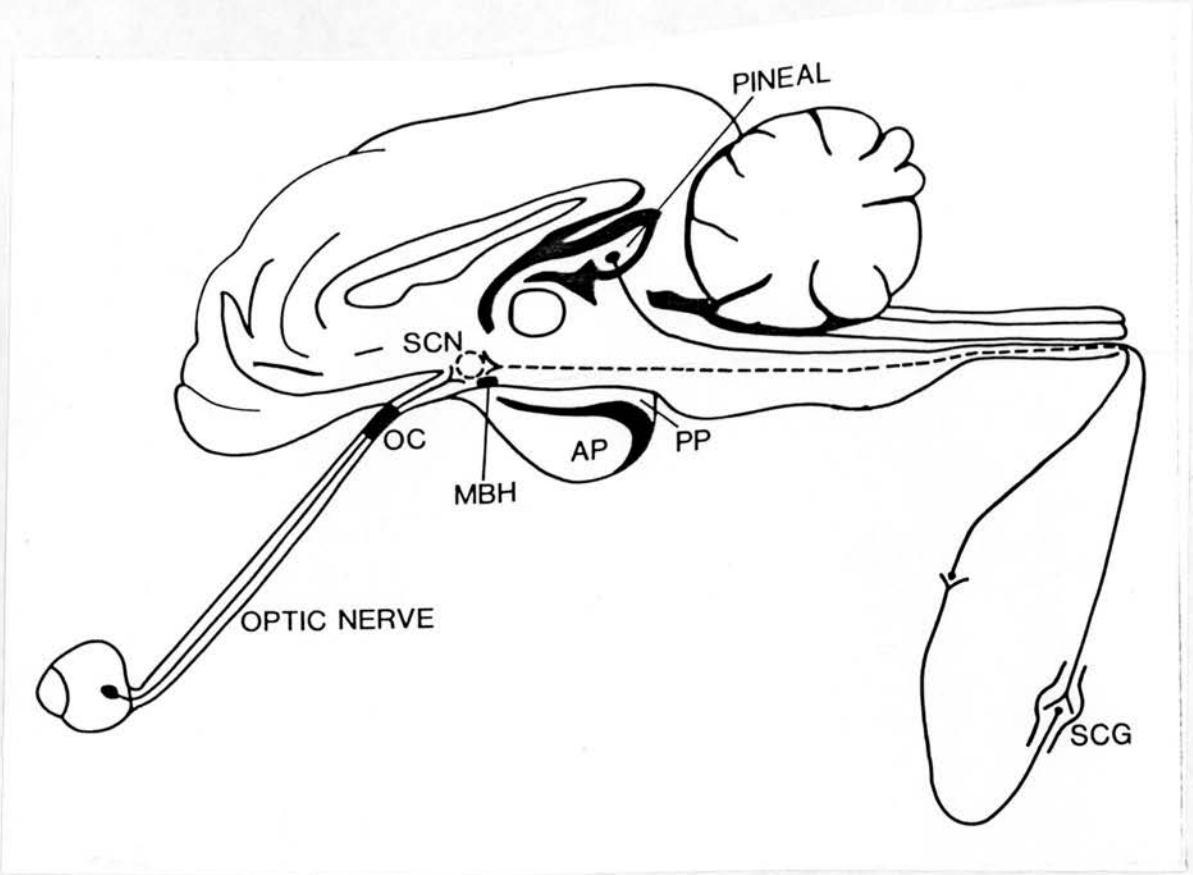


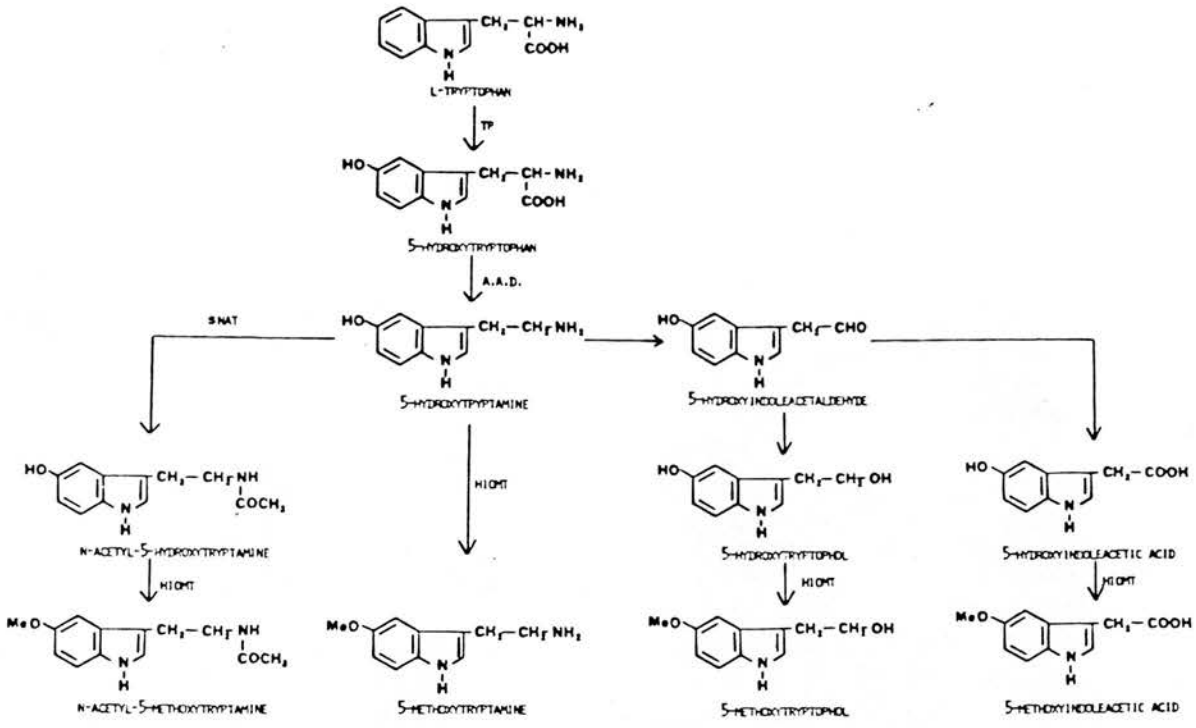
Fig. 1.5 Neural pathway by which light reaches the pineal. Light falling on the retina is transformed into a neural signal which is conducted to the hypothalamic suprachiasmatic nuclei (SCN). This signal is carried by unmyelinated nerve fibres which form the retino-hypothalamic tract (RHT). The RHT is contained within the optic nerve, but is independent of the primary visual tract. From the SCN, the photic information is conducted along a pathway which descends, via the spinal cord, to the pre-ganglionic neurones supplying the sympathetic superior cervical ganglion (SCG). The neural signal is reversed within the SCG, and the relase of norepinephrine (NE) by the post-ganglionic neurones which ascend from the SCG to the pineal is inhibited. The dotted lines indicate portions of the pathway which have not yet been anatomically identified.

1.2 PINEAL CHEMISTRY

The mammalian pineal gland contains a wide range of molecules. Quay (1974) lists the various classes of compound as: inorganic constituents, lipids, carbohydrates, amino acids, indoleamines, catecholamines, nucleotides and nucleic acids, proteins, peptides, pigments and "miscellaneous compounds". The chemistry of these principles is described by Quay (1974).

All the peptides isolated from the pineals of mammals have molecular weights <2000. To date, 12 pineal peptides have been described. While their names reflect their biological actions, their structures are not necessarily identical to those of their hypothalamic name-sakes. The peptides identified are: arginine vasotocin and lysine vasotocin (AVT and LVT; Milcu, Pavel & Neacsu, 1963; Pavel, 1978), gonadotrophin-releasing hormone (GnRH, LHRH; King & Millar, 1981; Piekut & Knigge, 1981), thyrotrophin releasing hormone (TRH; Youngblood, Hunn & Kizer, 1979), thyrotrophin inhibitory hormone (TRIH; Vriend, Hinkle & Knigge, 1980), pineal prolactin releasing and inhibitory factors (PPRF and PPIF; Chang, Ebels & Benson, 1979), pineal antigonadotrophin (PAG; Benson, Larson & Findell, 1981), threonylseryllysine (threonine-serine-lysine; Orts, Bruot & Sartin, 1980), somatostatin releasing inhibitory factor (SRIF; Dube, Le Clerc & Pelletier, 1975), oxytocin (Oxy; Dogterom, Suijdewint, Pevet & Bruijs, 1979) and arginine vasopressin (AVP, Dogterom *et al*, 1979).

The only other class of pineal hormone that has received a great deal of study is that of the indoleamines. Indeed, these compounds have received even more attention than the peptides, especially in regard to reproduction. The following section is therefore devoted to the chemistry of the indoleamines.



METABOLISM OF PINEAL INDOLES

Fig. 1.6 Synthesis of pineal indoleamines.

Abbreviations: TP, tryptophan hydroxylase;
SNAT, N-acetyl transferase;
AAD, aromatic animo acid decarboxylase;
HIOMT, hydroxindole-o-methyltransferase.
N-acetyl-5-methoxytryptamine = MELATONIN.

1.2.1 Indoleamines

Synthesis: Knowledge of the synthesis of the pineal indoleamines comes mainly from studies with rat pineal tissue. The amino acid L-tryptophan is the common precursor of all the indoleamines synthesised in the pineal and elsewhere in the brain. The first step in indoleamine synthesis is the hydrolysis of tryptophan to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. Pineal 5-HTP is unstable and is rapidly converted to serotonin (5-HT) by the enzyme aromatic amino acid-decarboxylase. The sympathetic fibres which innervate the pineal also contain large amounts of 5-HT (Wurtman et al, 1968) and this is likely to be another source of this hydroxyindole in the pineal. Of all the indoleamines found in the pineal, 5-HT is found in the highest concentrations.

Serotonin serves as a precursor for the synthesis of a number of methoxyindoles as shown in Fig.1.6. One of these methoxyindoles, N-acetyl-5-methoxytryptamine or melatonin, is the compound of central interest to this thesis. Its synthesis from 5-HT involves two enzymatic steps. Serotonin is first acetylated to N-acetyl-serotonin through the action of N-acetyl-transferase (SNAT; EC2.3.1.5). The N-acetyl-serotonin is then methylated by hydroxyindole-O-methyltransferase to form melatonin (MEL). Since the enzyme SNAT is specific to the synthesis of MEL, its activity has frequently been measured as an index of MEL synthesis (Klein, 1978).

Neural regulation of indoleamine synthesis: The synthesis and secretion of pineal indoleamines change in relation to the environmental LD cycle, e.g. maximal amounts of 5-HT are found in the pineal during the daily light phase, while maximal amounts of the methoxyindoles, such as MEL, are found during darkness (Quay, 1974; Rollag & Niswender,

1976; Kennaway, Frith, Phillipou, Matthews & Seamark, 1977; Leone, Silman, Hooper, Finnie, Carter, Edwards, Smith, Towell & Mullen, 1979; Cardinali, 1981).

Photic information from the environment is conveyed to the pineal through its sympathetic innervation. Light excites neurones in the SCN which transmit impulses to the SCG. These impulses cause an inhibition of the spontaneous discharges from the post-SCG sympathetic fibres which terminate in the pineal gland. During periods of darkness, these fibres are excited and release NE from their terminals. This NE in turn stimulates the pinealocytes (Newman, Taylor & Wilson, 1970; Nishino, Koizumi & Brooks, 1976; Semm & Vollrath, 1979). A daily rhythm in NE release in the region of the rat pineal has been recorded by Brownstein & Axelrod (1974).

The effects of NE on pineal metabolism have been most thoroughly studied with rat pineal organ culture systems using isotopically-labelled tryptophan as the precursor (Minneman & Wurtman, 1976; Klein, 1978). Although those studies were specifically designed to study the regulation of the synthesis of MEL, their results may be equally applicable to any of the other pineal indoleamines. During darkness, the NE released from the sympathetic nerve terminals reaches the pinealocyte by diffusion (Kappers, 1976). Here it binds to β-adrenergic receptors, causing an increase in intracellular cyclic-adenosine 3, 5-monophosphate (cAMP). The cAMP, acting as a second messenger, triggers off de novo protein synthesis, including that of the enzymes required for the conversion of tryptophan to 5-HT, and of 5-HT to the various methoxyindoles. The synthesis of N-acetyl-transferase is believed to be particularly dependent upon this NE stimulation for its synthesis (Klein, 1978).

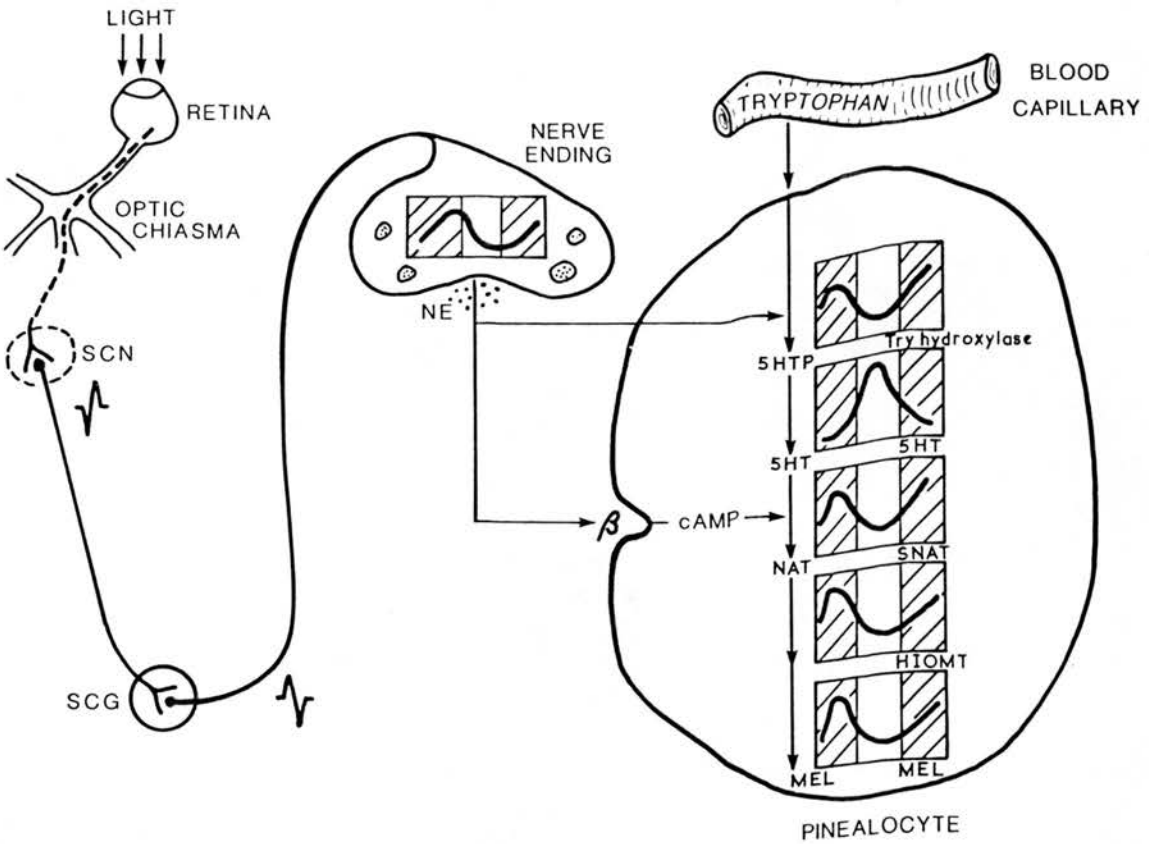


Fig. 1.7 Rhythm in the biosynthesis of melatonin. The enzymes involved in the various conversion steps are shown in Fig. 1.6. The production of the enzyme serotonin N-acetyl-transferase (SNAT) appears to be the rate-limiting step in the synthesis of melatonin.

The rhythmical synthesis of products in the MEL synthetic pathway are shown in Fig.1.7. It should be noted that while there is a diurnal variation in the blood levels of tryptophan (Wurtman, Rose, Chou & Larin, 1968), its uptake into the pinealocytes seems to be regulated by NE since it is possible to stimulate tryptophan uptake by the addition of NE to the medium of rat pinealocytes in culture (Wurtman, Shein, Axelrod & Larin, 1969).

As shown earlier in Fig.1.4, pineal rhythms persist during exposure to constant photoperiods, after blinding and following lesions which do not include the nerve tracts between the SCN and the pineal; the rhythms that persist still have a period close to 24 h, but they bear no relationship to the external LD cycle. Destruction of the nerve tracts linking the SCN and pineal results in a loss of all pineal rhythmicity. However, pineal indole metabolism is not necessarily abolished after such lesions, since NE may still reach the pineal cells from the original sympathetic nerve endings, or from surrounding nervous tissue. The lesion studies have led to the view that the pineal rhythm is generated from within the SCN; the SCN are now known to generate many other 24 h rhythms in mammals (Axelrod, 1978; Moore, 1978, 1979; Klein, Weller, Reppert, Tamarkin & Auerbach, 1979; Rusak & Zucker, 1979).

Pineal indole metabolism can also be altered by various pharmacological procedures. These methods involve drugs which either stimulate or block the βadrenergic receptors on the pinealocytes, or which block protein synthesis (Minneman & Wurtman, 1976; Smith, 1981). Drugs which interfere with SCN function can also be used to modify pineal activity (Zatz, 1979).

1.2.2 Route of Pineal Secretions

It is a matter of debate as to which compartment, the CSF or the blood, or both, the pineal secretes its principles into. The gland appears to be outside the blood-brain barrier, and it has no structures which might be associated with the transport of molecules, that extend into the 3rd ventricle (Reiter et al 1975; Trentini, Mess, de Caetani & Ruzsas, 1979; Cardinali, 1981). Indeed, cytological evidence suggests that the pineal cells release the contents of their secretory granules into the perivascular space (Kappers, 1971).

Measurements of the most commonly studied pineal compound, MEL, show that this putative hormone is found in both the CSF and the blood of several mammalian species (Man: Arendt, Wetterberg, Heyden, Sizonenko & Paunier, 1977; sheep: Rollag, Morgan & Niswender, 1977, 1978; rhesus monkeys: Perlow, Reppert, Boyar & Klein, 1981). Generally the amount found in the CSF is only a small fraction of that found in the blood; for example Rollag et al (1977, 1978) reported concentrations in the blood which were approximately 100 times those in the CSF.

The morphological and biochemical evidence thus implies that the blood receives the major proportion of pineal secretions. However, since it is widely believed that the sites of action of the pineal products probably reside within the brain (Demaine & Kann, 1979; Trentini et al, 1979; Reiter, 1980; Cardinali, 1981; Glass & Lynch, 1981, 1982), it could be argued that only small concentrations of the pineal hormones would be required for action; after all, the hormones would reach their intended sites rapidly, and without the dilution and metabolism that they would be subject to, if they entered the general circulation.

Quay (1973) has proposed that there may be a counter-current flow of blood within the great cerebral vein, whereby pineal hormones that enter capillaries within the pineal would have ready access to the intraventricular CSF without, or before, entering the systemic circulation. Retrograde blood flow has been shown to occur in several major blood vessels, including the great cerebral vein (Reiter et al, 1975).

Although there is evidence that MEL and other pineal Methoxyindoles are bound to blood plasma proteins (Cardinali, Lynch & Wurtman, 1972), the binding is apparently easily reversible. Indeed, Laud & Smith (1979) reported that these methoxyindoles were bound to high capacity, low affinity binding sites.

1.3 SEASONAL BREEDING IN MAMMALS

1.3.1 The phenomenon: The survival of most mammalian species of the temperate and polar regions relies upon their ability to produce their young at a time of year when the chances for survival of the young and their parents are maximal. Most mammals have thus adopted a strategy of reversible sexuality and fertility, and cycles of reproductive activity recur on an annual basis in a very reproducible manner. The fluctuations in reproductive competence are closely synchronised with the seasons and hence, the phenomenon is called seasonal breeding.

Climate, predation and availability of food are three factors which dictate the optimal time for parturition; Baker (1938) referred to these as the "ultimate causes" of seasonal breeding. How then, do mammals predict the time for the onset of breeding activity, given that, in most species, (1) the most propitious time for giving birth, and (2) the length of gestation are fixed?

In theory, the very same ultimate factors which determine the optimal time for giving birth could also serve as environmental

time-telling cues (zeitgebers) for the more proximate events leading to breeding and conception. In small mammals which have short gestation lengths, the ultimate factors are possibly the same as the proximate ones. However, in larger mammals, in which gestation can last for several months, factors which allow the anticipation of the time for breeding become necessary. These factors must be reliable so as not to cause a wastage of reproductive effort. Cyclic events in our solar system provide a stable cue in the form of seasonal changes in daylength. Most seasonal breeders appear to have adapted to respond to changes in the daily photoperiod in order to time not only their reproductive cycles, but also their cyles of wool growth, horn growth and appetite; these other cycles themselves contribute to the survival and reproductive success of individuals (Hoffmann, 1981a).

That the annual cycle in daylength could serve as a timing mechanism was first proposed by Schaffer (1907). Empirical proof for this idea was provided by Rowan (1925) who showed that the gonads of the Junco bird (Junco hyemalis) could be stimulated by exposure to artificially long days during the winter. The field vole (Microtus agrestis) was the first mammal in which daylength was shown to have effects upon the timing of breeding (Baker & Ranson, 1932). The list of photoperiodic mammals is now considerable, and includes the sheep (Clarke, 1981). In every species investigated, the effects of the natural photoperiod can be reproduced by artificial photoperiods; this allows the study of seasonal breeding under the controlled conditions of the laboratory.

Most small mammals have adapted to respond to long daily photoperiods for the induction of reproductive activity, whereas larger

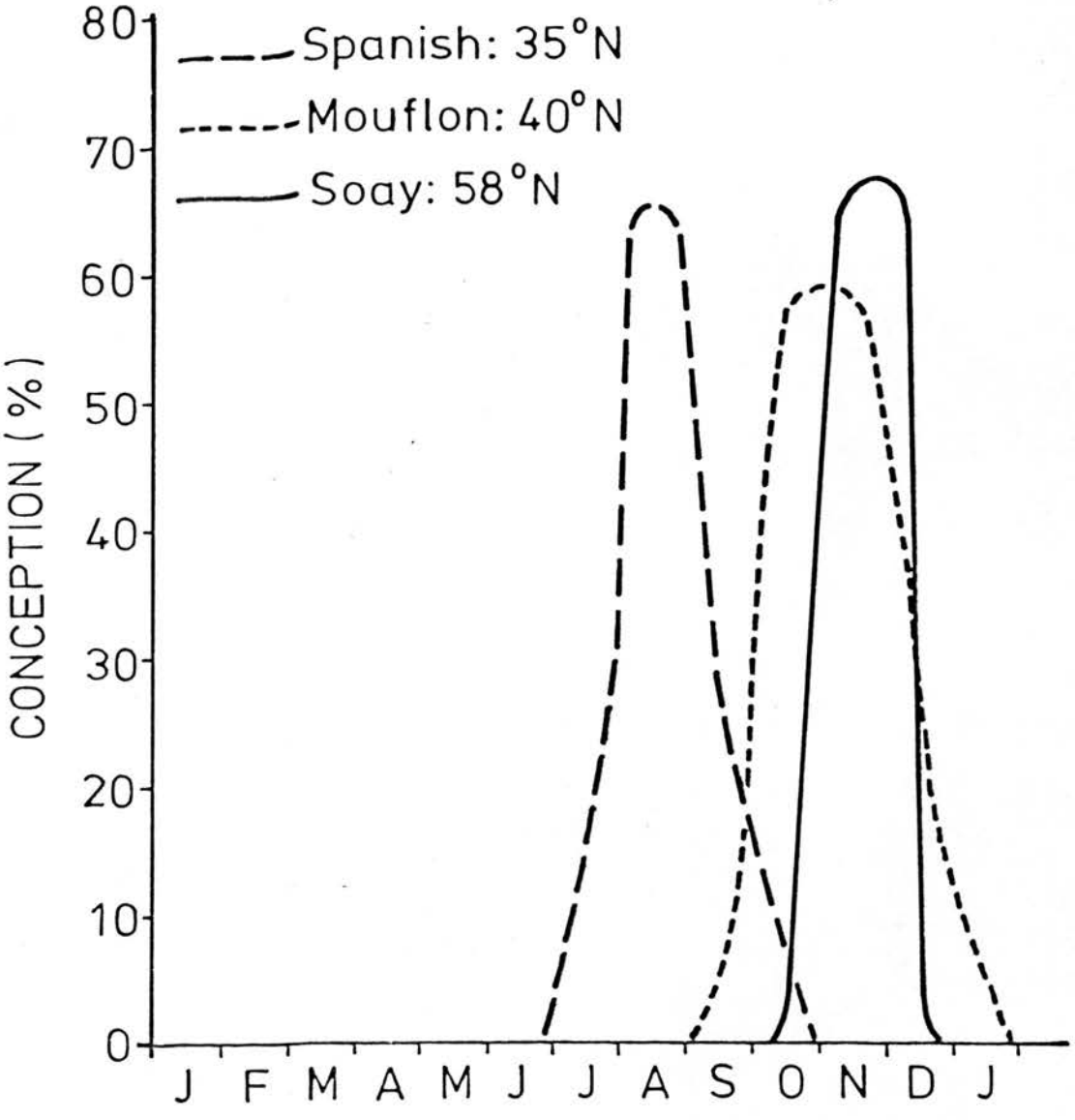


Fig. 1.8 Times of conception in three breeds of sheep originating from different latitudes (data from: Hafez, 1952).

ones such as sheep and deer have adapted to use a decrease in daylength as a signal to enter the breeding condition (Turek & Campbell, 1979; Clarke, 1981). Since the period of gestation varies from species to species, responses to either long daylengths or short daylengths, allows most species to produce their young during the spring and early summer months. The daily photoperiod also appears to have a role in timing the breeding seasons of mammals in which the proper timing of birth is achieved by delayed implantation (Clarke, 1981; Flint, Renfree & Weir, 1981; Hoffmann, 1981a).

1.3.2 Critical Daylength

Differences between species in their responses to daylength can be explained in terms of each species having its own particular threshold of response or critical daylength. Thus a long day species such as the golden hamster (Mesocricetus auratus) breeds when daylengths are in excess of 12.5 h (Gaston & Menaker, 1967), whereas a short day species such as the sheep breeds when daylengths fall below about 10.5 h (Hafez, 1952).

Considerable differences in critical daylengths also appear to exist between breeds of sheep originating from different latitudes, and this gives rise to breed differences in the periods of sexual activity, conception and birth. The rates of conception, with respect to time of year, in three breeds of sheep are shown in Fig.1.8. The Spanish-Piebald ewe from 35°N conceives between July and November (peak conception rate in August) and the Mouflon from 40°N conceives between September and January (peak in November). The Soay from 58°N has a much narrower period of conception, ranging from October to December (peak in November). Generally, those breeds from the higher latitudes have a shorter critical daylength and a correspondingly later breeding period; this adaptation allows them to produce their

young later in the year to correlate with the later occurrence of optimal conditions for the survival of the offspring and parents (Jewell & Grubb, 1974).

Differences in critical daylength might also occur between the sexes. Short (1973) suggested that such differences would be of adaptive advantage since the male mammal requires several weeks for spermatogenesis whereas the female requires only a few days for the reactivation of her ovaries to the point of ovulation. Thus, if the male had a different critical daylength from the female, the chances of fertile matings would be improved.

1.3.3 Measurement of daylength

Two hypothetical mechanisms by which animals might measure changes in daylength exist. The hour-glass hypothesis proposes that animals in some way count the number of hours of light or darkness each day. The ratio of light to dark is perhaps reflected by the amount of some chemical substance which accumulates to affect seasonal cycles (Hillman, 1979; Rusak & Zucker, 1979).

The second model for photoperiodic time measurement is the circadian model (Bünning, 1973). This hypothesis is based on the organism having an endogenous 24h rhythm in sensitivity to light or darkness. It is the coincidence of either light or darkness with the sensitive phase, which determines the photoperiodic response; the sensitive phase is, itself, entrained by the LD cycle. The majority of evidence suggests that mammals have a circadian basis to their measurement of daylength (Ravault & Ortavant, 1977; Turek & Campbell, 1979; Rusak & Zucker, 1979; Elliott, 1981; Elliott & Goldman, 1981; Hoffmann, 1981a; Rusak, 1981).

1.3.4 Photorefractoriness and Annual Rhythms

The relationship between photoperiod and breeding cycle is not a simple one. In many birds and mamamals, prolonged exposure to a particular photoperiod results in the development of insensitivity to that photoperiod, i.e. the animals become photorefractory. The length of exposure to a particular photoperiod before refractoriness to it occurs, varies between species. Usually, the refractory condition persists until the animal is exposed to a photoperiod different to that which induced the refractory condition in the first place. There are a few species, however, which do not strictly conform to this pattern (Turek & Campbell, 1979; Stetson & Tate-Ostroff, 1981).

Most small rodents become refractory to the gonado-inhibitory effects of short daylengths. These species redevelop their gonads during prolonged exposure to short days, but this redevelopment is characterised by its sluggishness, relative to the rapid development seen in response to long days (white-footed mouse: Johnston & Zucker, 1980b; golden hamster: Reiter, 1972; Stetson, Matt & Watson-Whitmyre, 1976; Stetson & Tate-Ostroff, 1981; Djungarian hamster: Hoffmann, 1981a-c).

Ferrets become refractory to the stimulatory effects of long daily photoperiods (Thorpe & Herbert, 1976), and so resemble many avian species (Hammer, 1968; Turek & Campbell, 1979). However, it seems that photorefractoriness in ferrets may not be of the usual type, since Thorpe & Herbert (1976) found that a minority of individuals could recover spontaneously from the refactory condition, i.e. without exposure to a different LD cycle. This type of photorefactoriness has also been recorded in at least one species of bird, the Japanese quail

(Coturnix coturnix japonica); Follett, Robinson, Simpson & Harlow (1981) reported that refractoriness in this species could be prevented by transferring birds to increasingly long daylengths.

Photorefractoriness occurs under both natural and artificial photoperiods and may be regarded as an adaptive safety mechanism. When it occurs to terminate breeding it may be considered to serve to prevent ferilisation late in the season, even though the "proximate factors" may still be favourable. Since gestation and incubation are of fixed duration, late breeding could result in the arrival of offspring when the "ultimate factors" are exhausted or unfavourable (Lofts & Murton, 1968). When it occurs to induce breeding, it may be considered as a mechanism which maximises the continuation of the species despite the apparently adverse conditions which may prevail. The latter case might, of course, be an indication that the animals have become responsive to some other environmental cue to time their breeding.

Some birds and mammals which have apparently become photorefractory, have been found to express seasonal reproductive and metabolic cycles under constant photoperiods. These cycles recur at approximately 12-monthly intervals and have therefore been termed "circannual rhythms" (Pengelley & Asmundson, 1971; Ducker, Bowman & Temple, 1973; Pengelley, 1974; Gwinner, 1981). Some authors argue that the circannual rhythm is endogenously generated, with changes in the LD cycle simply serving to couple the internal rhythm with the seasons (Pengelley, 1974; Gwinner, 1981).

1.3.5 Seasonal breeding in sheep

The best experimental evidence that photoperiod is the principal cue used by sheep for the timing of their breeding cycles comes from the studies of Marshall (1937) and Yeates (1949). Marshall transferred sheep from the northern hemisphere to the southern hemisphere and observed that the breeding season of the sheep was shifted by 6 months, corresponding to the season in their new environment. Yeates (1949) demonstrated that, irrespective of fluctuations in other environmental variables such as food and temperature, the sexual cycle of the sheep was principally responsive to manipulation of the photoperiod.

Seasonal reproduction in sheep has been the subject of several reviews (Hafez, 1952; Lincoln & Short, 1980; Karsch & Foster, 1981; Legan & Winans, 1981; Thimonier, 1981). While all breeds of sheep display seasonal cycles of breeding activity, it is in those from the higher latitudes that seasonal cycles are most pronounced.

Soay Sheep

The Soay breed is one of the most seasonal breeds of sheep. It originates from the remote islands of St. Kilda, several hundred miles off the north-west coast of Scotland (58°N). Its ancestors were introduced to St. Kilda by Norsemen during the Bronze Age (ca.5000 B.C.), and that neolithic stock is believed to have stemmed from the Mouflon sheep of the European Mediterranean Basin (Fraser Darling, 1974).

Climatic conditions are harsh, and vegetation sparse, on St. Kilda (Jewell, Milner & Morton-Boyd, 1974), and so seasonal breeding has been of paramount importance for the survival of the Soay sheep. Some features of the seasonal changes in temperature, daylength and vegetation on St. Kilda are shown in Fig. 1.9. Minimum temperatures occur in January (4°C) while maximum temperatures occur in July (13°C).

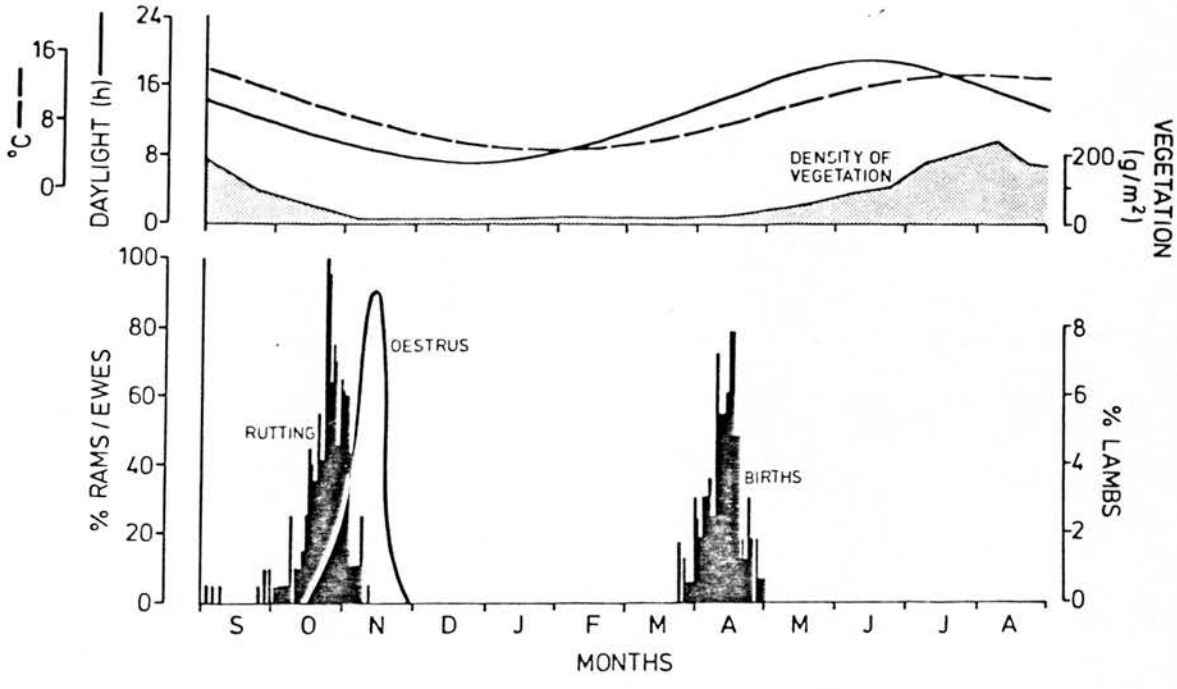


Fig. 1.9 The reproductive cycle of the Soay sheep in relation to changes in the external environment (temperature, day length and density of vegetation) on the Islands of St. Kilda (58°N). Data summarised from: Campbell (1974), Grubb (1974), Grubb & Jewell (1973), Jewell & Grubb (1974), Milner & Gwynne (1974) and H.M.S.O. Nautical Almanac (1976).

Precipitation is high (annual total 1200 mm), with snow or hail falling for up to 20 days each year. Strong winds occur during much of the year; gale force 8 winds have been recorded for about 70 days per year, and force 5-7 winds occur much more frequently (Campbell, 1974). The density of vegetation increases from about $10g/m^2$ in the winter months (December-March) to about 120 g/m^2 in July and August. The vegetation is most digestible between May and August (Milner & Gwynne, 1974).

During late September, the Soay rams on St. Kilda show the first signs of the annual rut. This behaviour, which is concerned with gaining access to ewes, reaches a peak in mid-October (Grubb, 1974). By this time a large proportion of the ewes will have started to show oestrus, and mating begins. Mating occurs in earnest during early to mid-November, by which time almost all of the ewes are showing oestrous cycles. The percentage of cycling ewes drops dramatically during the latter part of November, reflecting a high incidence of conception in the two previous weeks. The majority of conceptions occur during the second ovulatory cycle (Jewell & Grubb, 1974). Gestation in the Soay sheep lasts for a mean of 151.2 days (range: 148-155 days) and most births occur during the first half of April (Jewell & Grubb, 1974). The reproductive cycle of the Soay sheep on St. Kilda occurs with remarkable consistency each year. The distribution of the major events of the cycle are summarised in Fig. 1.9.

The endocrine events during the seasonal sexual cycle of Soay rams under natural daylengths has been studied in detail by Lincoln (Fig. 1.10). The secretion of luteinising hormone (LH) occurs episodically throughout the year, but the frequency of episodes, and amplitude of secretion, are greatest during the months of August and September, when daylengths are beginning to decrease. The blood concentrations of

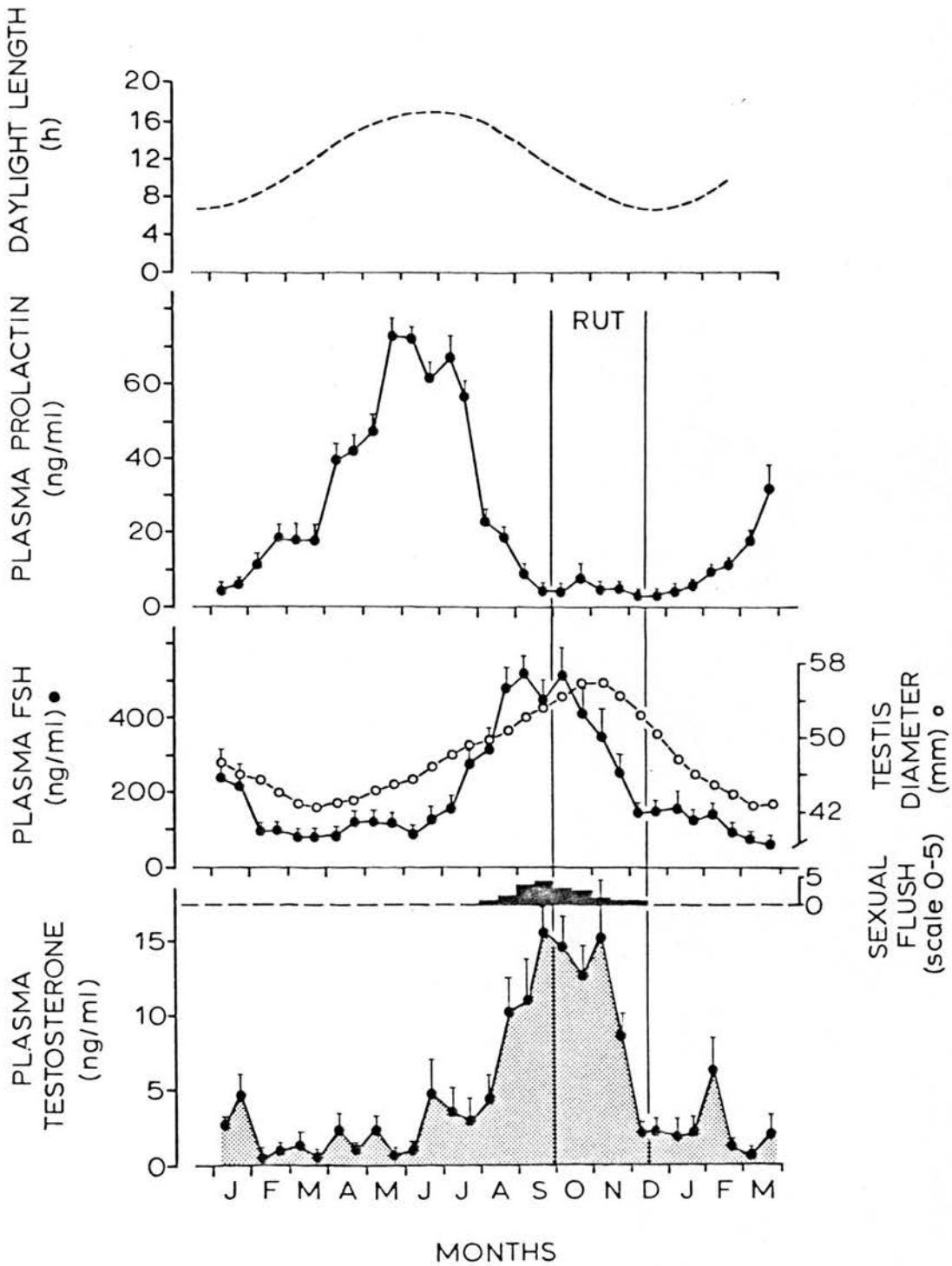


Fig. 1.10 Changes in the blood plasma concentrations of prolactin, follicle stimulating hormone and testosterone of 5 adult Soay rams (mean $\pm$ SEM) in relation to changes in the natural photoperiod at Edinburgh (56^{o}N). Changes in the diameter of the testes and sexual skin flush are also shown. (from: Lincoln & Short, 1980).

follicle stimulating hormone (FSH) begin to rise gradually from about July, reaching a maximum during September. The size of the testes increase from June onwards and reach a maximum diameter in October. The blood plasma concentrations of testosterone also begin to rise in June, reaching a peak in late September-early October; this peak corresponds with the onset of rutting behaviour and the appearance of an inguinal sexual skin flush. The skin flush is thought to be due to hyperaemia of the inguinal region (Lincoln & Davidson, 1977). Reproductive activity declines from November onwards, until the following autumn.

It should be noted that the changes observed in the pituitary-gonadal axis occur in advance of the longest and shortest days of the year (summer and winter solstices). In particular, the recovery of LH secretion and testicular size in advance of a decrease in the natural photoperiod, and the termination of reproductive activity while daylengths are still decreasing, might be considered to be photorefractory events.

The testes of Soay rams are three times as heavy during the breeding season as during the non-breeding season (Mortimer & Lincoln, 1982). Ultrastructural studies reveal only small changes in the Leydig cells between the breeding and non-breeding periods, despite a 30% reduction in their secretion of testosterone. Changes in Sertoli cell activity and seminiferous tubule diameter account for the large seasonal differences in testis size (Mortimer & Lincoln, 1982). The significance of the patterns of secretion of the gonadotrophins (LH and FSH) upon testicular steroidogenic and spermatogenic activity have been discussed by Lincoln & Short (1980).

Fig. 1.10 also shows seasonal changes in the blood concentrations of prolactin (PRL) in the Soay ram. The highest concentrations of PRL occur in the blood during the mid-summer months, when daylengths are

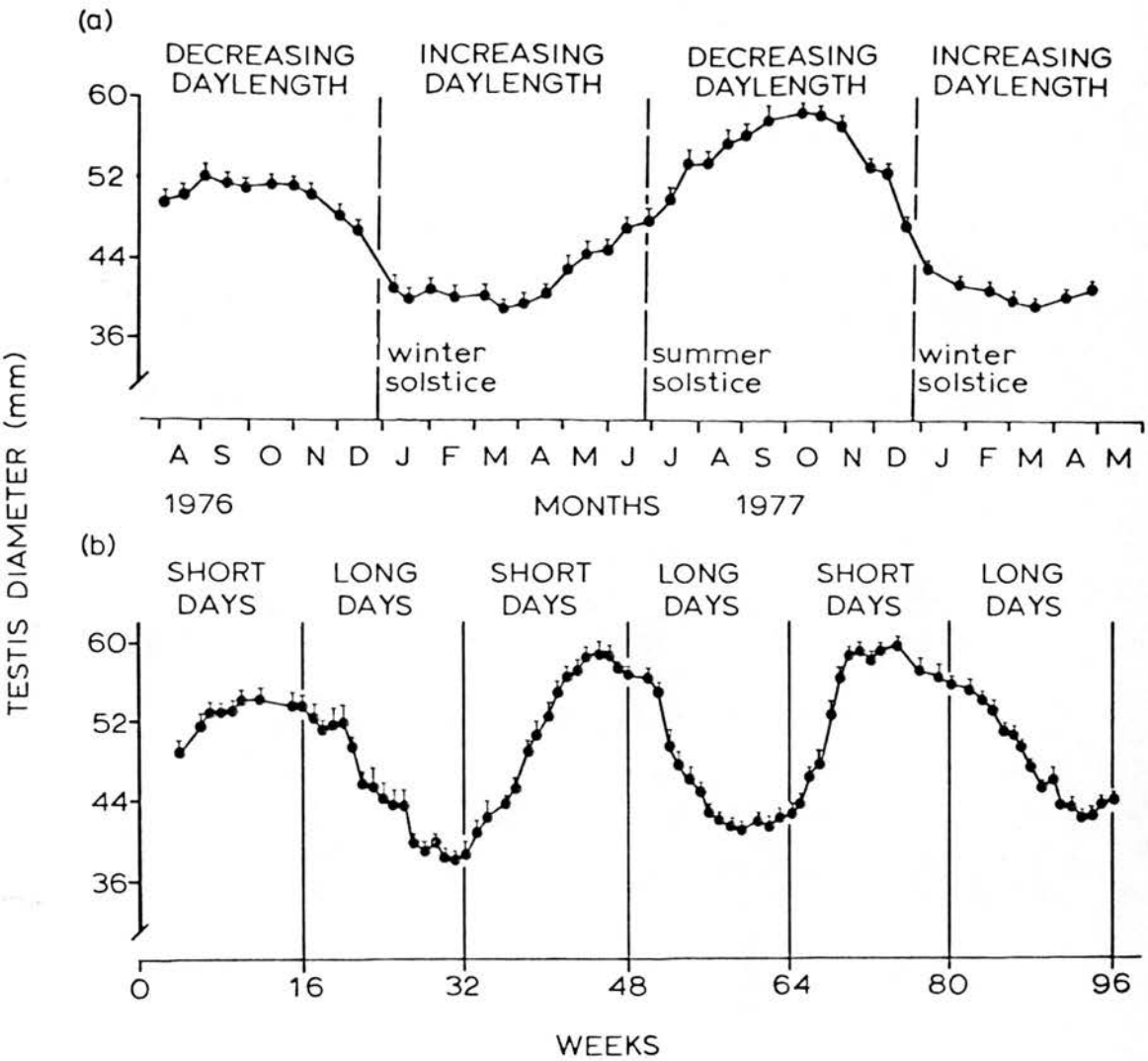


Fig. 1.11 Comparison of changes in the diameter of the testes (mean ± SEM) of Soay rams living (a) under natural lighting condition near Edinburgh (n = 14) and (b) under controlled lighting of alternating 16-week periods of short days (8L:16D) and long days (16L:8D). The rams in the first group had access to natural pastures, while those in the second group were maintained on a standard diet. (from: Lincoln & Short, 1980)

longest. Intermediate levels of PRL are found as daylengths begin to increase during the spring, and again, as daylength begins to decrease during the late-summer months. Minimal levels of PRL are found during the winter months. The secretion of PRL therefore shows a more-or-less inverse relationship to that of the gonadotrophins and to changes in testicular activity.

Cycles of sexual activity can be induced in the laboratory by exposing Soay rams to alternating 16 week periods of artificial long days and short days (Lincoln & Davidson, 1977). Several such artificially-induced testicular cycles, during which the rams received a constant level of nutrition, are shown in Fig. 1.11. Comparison with the natural cycles (Fig. 1.11) shows that the testicular responses were accelerated under the artificial photoperiods, due to the contracted cycle length (32 weeks vs. 52 weeks). Otherwise, the sequence and magnitude of changes in gonadotrophin and prolactin secretion and testicular activity remained unchanged under these artificial cycles (Lincoln & Short, 1980). From his detailed studies of gonadotrophin secretion under such artificial photoperiodic cycles, Lincoln deduced how the episodic release of hypothalamic gonadotrophin releasing hormone (GnRH) might control the secretion of LH and FSH by the pituitary (Lincoln & Short, 1980; Lincoln, 1981).

1.4 IMPLICATION OF THE PINEAL GLAND AND MELATONIN IN THE CONTROL OF SEASONAL REPRODUCTION IN MAMMALS

Many early studies with extracts of pineal tissue suggested that the pineal might have inhibitory effects upon the gonads (Kitay & Altschule, 1954). This concept was supported by the studies of Quay (1956) on the morphology of the pinealocytes of the white-footed mouse (Peromyscus leucopus) during various stages of its reproductive cycle. Quay reported large, and histologically active, pineals during the

non-breeding period compared with the breeding period. Other indirect evidence for pineal involvement in seasonal breeding comes from the data reviewed by Ralph (1975) showing that mammalian species from the higher latitudes had larger pineals than species from the tropical and equatorial regions; it is the species from the high latitudes that have the most marked breeding seasons.

Removal of the pineal (PINX), or interfering with structures involved in the transfer of photic information to the pineal (e.g. superior cervical ganglionectomy, SCGx), alters the ability of many mammals to respond to changes in the photoperiod for the timing of their reproductive cycles. Thus, in long day species such as the golden and Djungarian hamsters, ferrets, and voles, PINX or SCGx prevent the gonadal involution normally induced by short daylengths or by blinding (Hoffmann & Reiter, 1965; Hoffmann, 1974; Farrar & Clarke, 1976; Thorpe & Herbert, 1976). On the basis of such results from long day species, several authors promoted the view that the pineal exerted inhibitory effects upon reproduction (Turek & Campbell, 1979; Reiter, 1980). Since these effects were not manifest during long days, it was suggested that such species were "physiologically pinealectomised" under long daily photoperiods.

If the pineal really acted to inhibit reproduction during short days, by secreting more antigonadotrophin for example, an opposite hypothesis would need to be invoked to explain breeding activity induced by short daylengths in species such as the sheep and deer. Moreover, in at least two long day breeders, the ferret and Djungarian hamster, the pineal also appears to be involved in the stimulatory effects of the photoperiod upon reproduction. For example, Herbert

(1969, 1971) found that SCGx or PINX ferrets were unable to advance the onset of oestrus in response to long days and Hoffmann & colleagues found that testicular redevelopment under long days was delayed in PINX Djungarian hamsters (Hoffman & Kuderling, 1975, 1977; Brackmann & Hoffmann, 1977). These observations thus argue against the anti-gonadotrophin hypothesis.

A more plausible hypothesis is that the pineal is necessary for the transduction of photoperiodic effects, and thus for the synchronization of reproductive activity with the prevailing season. In support of this idea, Herbert (1972) and Herbert, Stacey & Thorpe (1978) observed in ferrets kept under natural photoperiods, that PINX prevented neither the onset nor the termination of oestrus; however, the oestrous cycles of these animals eventually became asynchronous with the photoperiod.

The foregoing suggests that the pineal is involved in the temporal control of seasonal reproduction. So, how are its effects conveyed to the hypothalamo-hypophyseal-gonadal axis? A humoral mechanism is almost certainly involved, and both the pineal indoleamines and peptides have been suggested as candidates for this role (Reiter, 1980). The indoleamine most commonly suggested to fit this role has been MEL (Cardinali, 1981), while AVT is the most frequently suggested peptide (Pavel, Luca, Calb & Goldstein, 1979).

There is some tentative evidence that AVT might modify gonadotrophin secretion (Pavel, 1978; Pavel *et al*, 1979; Reiter, 1980; Benson *et al*, 1981). AVT concentrations in the pineal and CSF increase during darkness, in the same way as those of MEL. Since the administration of MEL induces AVT release *in vivo* (cats: Pavel *et al*,

1973), it has been suggested that this peptide might be the important pineal hormone, with the indoleamines simply acting as local peptide-releasing factors (Pavel et al, 1979; Reiter, 1980; Benson et al, 1981).

The evidence that MEL might be responsible for mediating the effects of photoperiod upon reproduction stem from studies involving the injection or implantation of this putative pineal hormone. The two methods give different results because in the first (injection) method, the MEL is rapidly cleared from the body, whereas the second (implantation) method results in a sustained elevation of MEL concentrations.

Several studies involving MEL implantation suggested that this indoleamine inhibited reproduction. For example, MEL implantation in male short-tailed weasels during the winter, prevented testicular recovery during subsequent exposure to long days; implantation during the summer resulted in testicular involution, despite the stimulatory long daily photoperiod (Rust & Meyer, 1969). Hoffmann (1978) made a similar observation in the Djungarian hamster; testicular development was delayed in hamsters implanted with MEL during the winter and subsequently exposed to artificial long daylengths. In the golden hamster, testicular regression was induced by MEL implants during long days (Turek, Desjardins & Menaker, 1976a), and implantation with MEL during short days delayed the development of the gonads during subsequent exposure to long days (Turek, Desjardins & Menaker, 1975a). When Soay rams were implanted with MEL and challenged with a period of short days, the implanted rams showed a very marked delay in the recrudescence of their gonads, compared with the control rams (Lincoln & Almeida, 1982). Thorpe & Herbert (1976) also observed antigonadal effects of MEL, albeit by injection; ferrets thus treated showed premature anoestrous during stimulatory long days.

In contrast, other studies have shown that MEL may actually promote reproductive activity. In sexually active adult golden and Djungarian hamsters, MEL implants prevent gonadal involution upon exposure to short days (Hoffmann, 1974; Reiter, Vaughan, Blask & Johnston, 1974; Turek et al, 1976a). If the animals are implanted with MEL after the testes have regressed under short days, premature redevelopment occurs (Turek & Losee, 1978).

The above results suggest that MEL implants counteract, or interfere with, the normal effects of the photoperiod upon gonadal activity. This conclusion is supported by the discovery that reproductive events which are photo-independent, for example, spontaneous recovery from the inhibitory effects of a particular photoperiod, are not affected by MEL implants; thus short day refractoriness in the Djungarian and golden hamsters cannot be prevented by MEL treatment (Hoffmann, 1973; Turek & Losee, 1978). Puberty in the golden hamster also occurs independently of the photoperiod (Reiter, Sorrentino & Hoffmann, 1970) and pre-pubertal treatment with MEL is without effect in this species (Turek, 1979a). In contrast, Brackmann (1977) showed that implantation with MEL caused a delay in the sexual maturation of Djungarian hamsters; pubertal development in this species is regulated by the photoperiod (Hoffmann, 1978). It is also pertinent to mention here that the effects of MEL implantation are more profound in photoperiodic mammals than in non-photoperiodic species (Turek, Desjardins & Menaker, 1976a).

The main criticism aimed at the experiments in which MEL implants have been used, is that the treatment results in an unphysiological situation, in which MEL levels are elevated throughout the day, rather than during periods of darkness only. This criticism has been overcome by the use of MEL injections, given at specific times of the day. Tamarkin, Westrom, Hamill & Goldman (1976) and Tamarkin, Lefebvre,

Hollister & Goldman (1977) found that single daily injections of MEL given near the end of the light period of a normally stimulatory long day, caused gonadal regression in male and female golden hamsters. However, a similar injection given earlier in the day did not cause gonadal regression, i.e. hamsters respond differently to MEL at different times of the day, suggesting that the pattern of MEL delivery, and thus presumably its secretion, is critical in determining its effects upon reproduction. Timed injections of MEL in golden hamsters have also proved capable of inhibiting testicular regression induced by short days (Turek & Pappas, 1980). Thus, MEL itself appears to modulate the photoperiodic signal. Both the amount and the temporal pattern of its availability appear to be important in determining the photoperiodic response. Lastly, as with implanted MEL, timed injections of MEL are only effective in altering photo-dependent changes in reproductive activity. For example, MEL injections in golden hamsters fail to prevent spontaneous gonadal recovery during short days (Bittman, 1978; Bittman, Goldman & Zucker, 1979), or of attainment of sexual maturity (Rissman, 1980).

Additional evidence that MEL might play a role in seasonal reproduction comes from observations in sheep and hamsters: the pineal content of the golden hamster varies throughout its seasonal reproductive cycle (Rollag, Panke & Reiter, 1980), and studies of the blood concentrations of MEL in sheep reveal significant changes in amplitude and temporal pattern which can be correlated with photoperiod and reproductive status (e.g. Rollag, O'Callaghan & Niswender, 1978; Arendt, 1978; Arendt & Symons, 1981).

It has been argued that MEL is not the pineal compound that modulates the photoperiodic responses of mammals, but rather that it is a local factor acting upon the pineal itself to release other pineal

substances such as AVT (Reiter, et al., 1974; Panel et al., 1979). This suggestion is refuted on the basis that MEL has similar effects on reproduction even after PINX (Hoffmann & Kuderling, 1977; Turek, 1977; Tamarkin & Goldman, 1977a; Goldman, Hall, Hollister, Roychoudhury, Tamarkin & Westrom, 1979). The effects of MEL therefore appear to be independent of the pineal gland itself. It is interesting however, that Bittman & Zucker (1981) found in the golden hamster, that re-sensitization by long days following short day refractoriness, can be prevented by PINX. They proposed that the pineal might secrete some other factor which restores brain sensitivity to both the photoperiod and MEL.

The site of MEL action remains elusive. Membrane and cytosol receptors for MEL have been identified in specific brain areas such as the medial basal hypothalamus (Cardinali, Vacas & Boyer, 1979), the pineal gland itself (Cardinali & Vacas, 1981; Grota, Holloway & Brown, 1981) and peripheral tissues such as the liver and reproductive tract (Cardinali, 1981; Cardinali & Vacas, 1981). The brain receptors for MEL are reported to show different capacities for the hormone at different times of the LD cycle (Cardinali & Vacas, 1981).

It has been reported that the effects of MEL are altered in golden hamsters with lesions placed in their SCN (Bittman, Goldman & Zucker, 1979; Rusak, 1980). This observation is interesting in view of a recent report that the effects of MEL implants upon reproduction in the white-footed mouse are most effective when placed in the medial pre-optic and supra-and retrochiasmatic nuclei of the hypothalamus (Glass & Lynch, 1981,1982). These studies therefore suggest that MEL might act to alter the time-measuring ability of mammals, through its actions on the central circadian rhythm generator, the SCN. The SCN have already been strongly implicated in the control of seasonal breeding in sheep and other mammals (Domanski, Przekop & Polkowska, 1980; Elliott &

Goldman, 1981).

It will be recalled that changing patterns in GnRH secretion are believed to cause the downstream changes seen during seasonal breeding cycles (Lincoln & Short, 1980; Goodman & Karsch, 1981). Studies by Demaine & Kann (1979,1981) indicate that MEL might act to alter the electrical activity of the hypothalamic neurones responsible for secreting GnRH. In vitro studies on the isolated, perifused medial basal hypothalamus also suggest such an action for MEL (Kao & Weisz, (1977).

Martin & Klein (1976) observed that MEL inhibited the neonatal rat's pituitary response to GnRH, in vitro, suggesting that the gonadotrophs might also be a site of MEL action. Similar in vivo data were reported for mature and immature dogs (Yamashita, Mieno, Shimizu & Yamashita, 1978; Mieno, Yamashita, Iimori & Yamashita, 1978). Tests in two other species, however, failed to support this idea (neonatal golden hamster: Bacon, 1982 ; Sattler & Martin, 1981; adult ewes: Symons & Arendt, 1982).

1.5 AIMS OF PRESENT STUDIES

There is a well-pronounced seasonal cycle in the reproductive activity of the Soay sheep (Jewell & Grubb, 1974; Lincoln & Short, 1980) and recently, much detailed information on the photoperiodic control of gonadotrophin secretion and testicular activity in this breed has been collected (Lincoln & Short, 1980). In addition, the photoperiodic response of Soay rams has been shown to depend upon an intact pineal gland (Lincoln, 1979a,b). With the availability of sensitive assays for the detection of MEL in the blood, it became feasible to investigate the role of this putative pineal hormone in the control of seasonal breeding in the Soay sheep. As part of the present research project, a radioimmunoassay for MEL (Rollag & Niswender, 1976) was established, and measurements of MEL were made in the blood plasma

of Soay rams exposed to a variety of photoperiods. This allowed correlations between patterns of MEL secretion and reproductive status to be investigated.

CHAPTER 2

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

2.1 ANIMALS : All the studies described in this thesis were carried out on rams of the Soay breed of sheep (Fig. 2.1). This breed was chosen because it shows pronounced seasonal changes in its reproductive physiology and behaviour (Grubb & Jewell, 1973; Lincoln & Davidson, 1977) ; futhermore, these changes can be induced by changes in the artificial photoperiod in the laboratory (Lincoln & Short, 1980). The origin and ecology of the Soay breed has been reviewed by Jewell, Milner & Morton-Boyd, (1974).

The rams were obtained as lambs from either the existing flocks held on the farms of the Animal Breeding Research Organization (ABRO) at Roslin, near Edinburgh or from a commercial source (P. Mapson, Cambridge). They were either maintained under natural photoperiods and diets supplemented with hay and turnips, or in light-proof sheds with artificial light-dark cycles. The rams held indoors were fed a formulated pelletted diet (ABRO, AA6), supplemented with hay.

The artificial daylight in the light-proof sheds was provided by fluorescent strip lights (Mazda) which gave approximately 160 Lux at the level of the sheep's heads. The artificial photoperiods the rams were normally exposed to consisted of alternating 16 week periods of either long daily photoperiods (16 hours light : 8 hour darkness, 16L:8D) or short daily photoperiods (8 hours light : 16 hours darkness 8L:16D). The changes from one photoperiod to another were abrupt and were achieved by altering the timing of "lights out" by 8 hours. Occasions when such schedules were not employed are specificially referred to in the text.

The temperature in the light-proof sheds was not controlled, but

Fig. 2.1 An adult Soay ram living under natural conditions on the field station belonging to the A.R.C. Animal Breeding Research Organization, Roslin, Midlothian (May, 1982).

supplementary heating was provided during the winter months to reduce day-night fluctuations in temperature.

The rams held in the sheds were individually penned, but were in visual, olfactory and auditory contact with each other. They were fed once daily between 08.00 and 09.00 hours. Water was available ad lib

The day-to-day care of the rams was provided by Norah Anderson.

2.2. Measurement of testis size and sexual flush

The shortest diameter of each testicle was measured, within the scrotum, using Vernier callipers (Lincoln & Davidson, 1977).

Hyperaemia of the inguinal skin, here referred to as the "sexual flush", was scored on an arbitrary scale of 0-5; the higher the flush score, the greater the degree of hyperaemia (Lincoln & Davidson, 1977). Intense sexual flushes were found to correspond with high blood levels of testosterone (Lincoln & Davidson, 1977).

2.3. BLOOD COLLECTION FROM SHEEP

2.3.1 Venepuncture: Single 5ml blood samples were collected by venepuncture of the jugular vein, using a heparinized (Weddel Pharmaceuticals, London) evacuated glass tube, fitted with a "Vacutainer" needle of 21G gauge (Beckton Dickinson USA). When serum samples were required, heparin-free tubes were used.

2.3.2 Cannulation: When blood samples were required at frequent intervals (e.g. every 20 minutes, or hourly), cannulae (Braunula Luer, Armour Pharmaceuticals) were inserted into a jugular vein of the rams. The area surrounding the point of insertion of the cannula was first shaved and swabbed with a disinfectant (thiomerosal in alcohol; Royal Infirmary of Edinburgh Pharmacy). The position of the cannula was maintained with 2-3 sutures placed through the skin of the neck. Connection of the cannula to a 3-way tap was made by means of a length of manometer tubing (Portex Ltd., Hythe, Kent). In this way the animals could be sampled with minimum disturbance. The cannulae were

inserted at least 16 h prior to the start of the blood sampling. They were kept patent by flushing through with heparinized saline (5000 IU heparin/litre 0.9% sterile saline). 3-4 ml samples of whole blood were withdrawn on each occasion and replaced with a similar volume of heparinized saline. Cannulae were repaired or replaced whenever feasible, and during darkness, this was done by using a low-intensity hand-held torch which emitted a point source of light that could be directed at the jugular vein. Care was taken not to shine the light into the eyes of the sheep.

2.3.4. Separation and storage of blood plasma and serum : Blood plasma was obtained by centrifuging the heparinized blood samples at 2000g for 30 minutes, and transferring the clear plasma supernatant into plastic vials (Sarstedt, Leicester). Blood from which the serum was to be harvested was allowed to clot for at least 16h at 4°C before centrifugation and aspiration of the supernatants into plastic vials. The plasma and serum samples were stored, capped, at -20°C until required for the assay of hormone and electrolyte concentrations and antibody titres. Anti-biotics were not added to any of the samples.

2.4. SUPERIOR CERVICAL GANGLIONECTOMY (SCGx)

The SCG were surgically removed from both sides of the neck by Dr. G.A. Lincoln, following the procedures described by Appleton & Waites (1959). The operations were conducted in partially sterile conditions, under fluothane/nitrous oxide oxygen (British Oxygen Co.) anaesthesia. The rams received 1ml (i.m.) of the antibiotic Streptopen (Glaxo) for 1 week after the SCGx. Rams which received the sham operation were similarly treated.

The SCGx rams all showed the symptoms of Horner's Syndrome (ptosis of eyelids, constantly warm horns due to hyperaemia of horns, dry nostrils) for up to 4 years after surgery (Lincoln, Klandorf & Anderson, 1980).

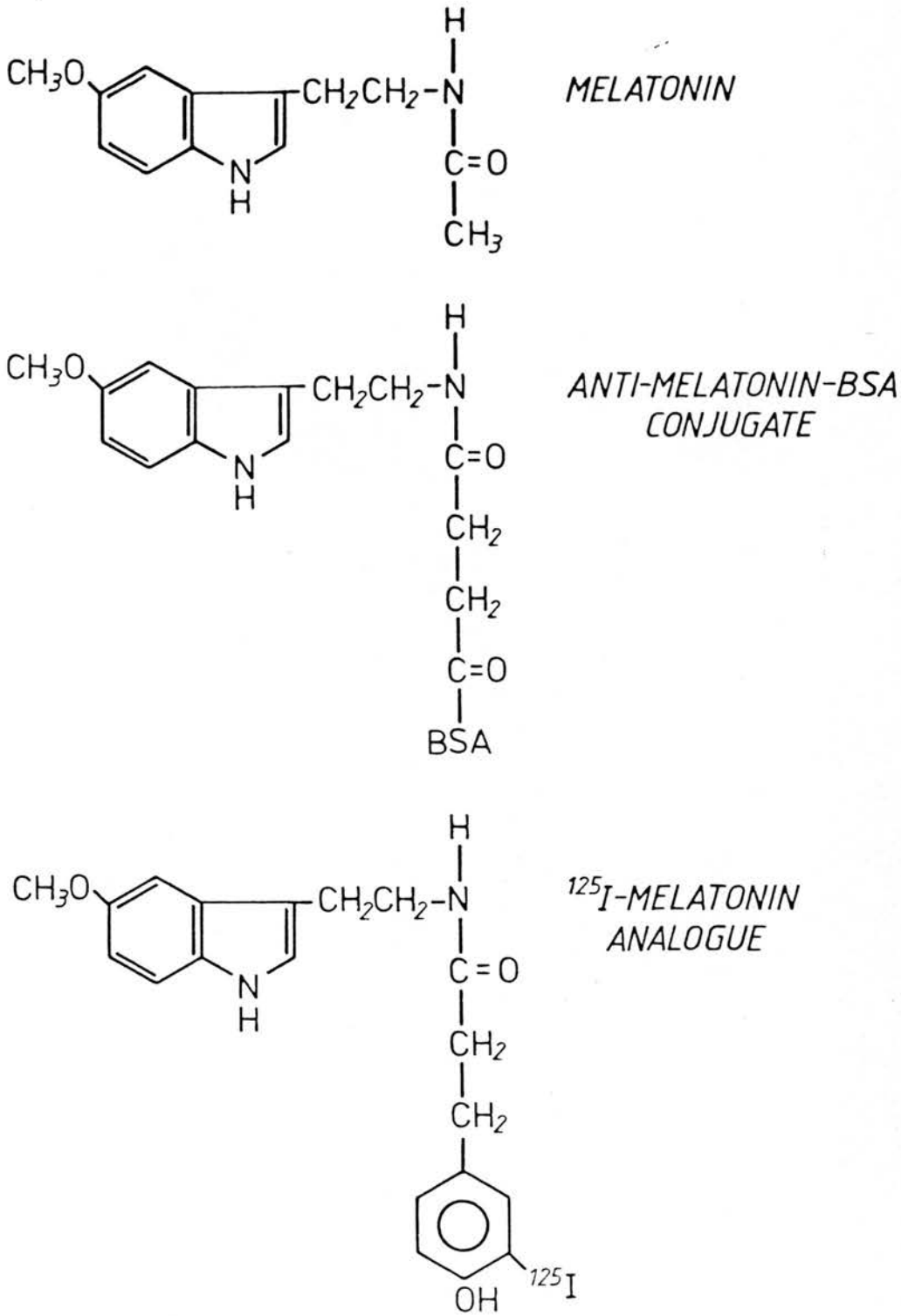


Fig. 2.2 Chemical structures of (top to bottom): melatonin (MEL), the conjugate used by Rollag & Niswender (1976) to raise the antiserum used for the RIA of MEL in these studies, and the ^{125}I-MEL analogue used in the MEL RIA.

2.5. RADIOIMMUNOASSAYS

2.5.1 Radioimmunoassay of Melatonin

Blood plasma concentrations of MEL were measured by the RIA method described by Rollag & Niswender (1976), after slight modifications. Initial attempts to set up the assay described by Arendt, Wetterberg, Heyden, Sizonenko & Paunier (1977) failed.

The radioimmunoassay (RIA) of a compound requires a specific high-titre, high-affinity antibody against the compound of interest (Skelly, Brown & Besch, 1973; Hunter, 1978; Hurn, 1974; Pratt, 1978). Melatonin (N-acetyl-5-methoxytryptamine, MW 232; Fig. 2.2) is not inherently antigenic but can be made immunogenic by conjugating it, or a closely related compound, to a large protein (e.g. BSA, thyroglobulin etc). Due to its small size, the production of specific antibodies to MEL has proved particularly difficult.

Radioimmunoassay also requires a radioactively-labelled form of the substance to be measured (Yalow & Berson, 1968). Hunter (1974) has discussed the need for pure antigens which can be labelled to high specific activities in order to achieve specificity and sensitivity. The size and structure of native MEL has been a limiting factor in the production of tracers with high specific-activities. For example, ^{3}H substitution can only be made in one position of the MEL molecule and the highest specific-activity achieved is in the order of 30 Ci/mmol, compared with 100 Ci/mmol for other haptens such as testosterone and progesterone. Hunter (1974) and Pratt (1978) describe many advantages of using ^{125}I-labelled antigens instead of ^{3}H-antigens; the chief advantages are convenience, precision and sensitivity. Direct radioiodination of the MEL molecule is not possible however, and while it is possible to successfully iodinate MEL-BSA conjugates, such tracers fail to bind to the antisera (O.F.X. Almeida, unpublished

observations; J. Arendt, personal communication). This problem presumably arises from the fact that the affinity that the antibody shows is greater for the bridge through which the hapten is attached to the carrier protein than for the hapten ("bridge-binding"; Hunter, Nars & Rutherford, 1975; Nordblom, Webb, Counsell & England, 1981). Rollag & Niswender (1976) synthesized an analogue of MEL, which was suitable for radioiodination, by conjugating the ester N-succinimidyl-3-(4-hydroxyphenyl)-propionate to 5-methoxytryptamine.
Their method is based on the "conjugation- labelling" technique of Bolton & Hunter (1973).

Initial assays : Initially, an RIA using ^{3}H-MEL (New England Nuclear, GmBH; 26Ci/mmol) was established using an antiserum raised against N-acetyl-5-methoxytryptophan-BSA (Rabbit K244 : Arendt et al., 1977; K244 obtained courtesy of Dr. J. Arendt, Guildford, Surrey). The sensitivity of that assay was 10pg/tube. However, K244 was not available in sufficient quantities for routine determinations and an unsuccessful attempt was then made to set up a sensitive RIA using a different antiserum provided by Dr. J. Arendt (Sheep B31; N-acetyl-5-methoxytryptophan-thyroglobulin). Both these earlier methods originally employed an ammonium sulphate precipitation step to separate bound from free fractions at the end of the assay, and the free fraction was counted. A more convenient method whereby the bound pellet was re-dissolved in methanol and counted in the assay tube, after the addition of 0.4% Butyl-PBD/ Toluene (Koch-light) scintillant was developed.

Final assay : Since an assay with high through-put and sensitivity was required for these studies, the system of Rollag & Niswender (1976) was eventually adopted, with some modifications as described below.

Antiserum

The antiserum (R1055), provided by Professor G.D. Niswender

(Colorado), was raised in rabbits against a conjugate of N-succinyl-5-methoxytryptamine and BSA (Rollag & Niswender, 1976; see Fig. 2.2). The specificity of this antiserum has been described by Rollag & Niswender (1976). The only further test of specificity conducted was with the compound O-Acetyl-5-methoxytryptophol (Smith, Francis, Leone & Mullen, 1980; synthesized by Dr. R.F. Seamark by acetylating N-acetyl-serotonin with acetic anhydride). A 100% cross-reactivity was obtained with this compound. However, its immunoreactivity could be reduced to undetectable levels by extracting it into 0.1M NaOH buffer (pH13). Subsequent studies using this extraction indicated that this compound is not normally present in sheep plasma (day or night). To date, O-Acetyl-5-methoxytryptophol has only been identified in the rat pineal gland (Balemans, Smith & de Reuver, 1981), and it seems that it is unlikely to occur in the plasma due to its susceptibility to hydrolysis (Dr. I. Smith, personal communication).

Synthesis of analogue of melatonin for radioiodination

The analogue, N-succinimidyl-3-(hydroxyphenyl)-propionate (Fig.2.2) was synthesised according to the method of Rollag & Niswender (1976) and Dr. M.D. Rollag (personal communication). All the reagents used were of analytical grade and, unless specified, were obtained from BDH Chemicals. Anhydrous dioxane was prepared by mixing 100 ml dioxane with 10 g of Grade 4A molecular sieves in a sealed container for 1 week.

Solution 'A' was prepared by dissolving 1 mmole (190 mg) of 5-methoxytryptamine (Sigma) and 1 mmol (238 ul) of tri-N-butylamine (Koch-Light) in 15 ml dry dioxane. (The tri-N-butylamine serves as a catalyst). Solution 'B' was prepared by dissolving 1 mmole (263mg) N-succinimidyl-3-(4-hydroxyphenyl)-propionate in 15 ml dry dioxane. Solutions 'A' and 'B' were mixed, and left in a shaking water-bath at 22°C, for 12hr in darkness. The reaction mixture was then centrifuged

for 0.5hr at 2000g. The pellett was discarded, and the supernatant was transferred to a separating funnel and washed with 20ml 0.1M $NaHCO_3$ (pH 8-9) for 1 hr, so as to hydrolyse any unreacted N-succinimidyl-3-(4-hydroxyphenyl)-propionate. The melatonin analogue, together with unreacted 5-methoxytryptamine and tri-N-butylamine, was extracted from this mixture with 50ml ethyl acetate. The ethyl acetate extract was washed 3 times with 10ml 0.1M $NaHCO_3$ (pH 8-9) and 3 times with 0.1N HCl (adding extra ethyl acetate as appropriate), so as to partition the analogue into the organic phase, and the 5-methoxytryptamine and tri-N-butylamine into the acidic aqueous phase. The solvent was removed using a rotary evaporator and the resulting white oil stored in 5ml methanol. The concentration of this 'stock solution' was determined by measuring its optical density at a wavelength of 279nm (extinction coefficient at 279nm=5000; M.D. Rollag, personal communication). The analogue was synthesised on two occasions with yields of 40.3% and 57.3%; a 100% yield would have provided 338mg (1 mmole) of the analogue. The stock methanolic analogue solutions were stored at -20°C, and appropriately diluted with methanol to give a 'working solution' containing 0.5 mg analogue/ml methanol when required for radioiodination.

Radioiodination of Melatonin Analogue

The MEL analogue was radioiodinated according to the method of Rollag & Niswender (1976), with slight modifications.

To 5 ug (15 nmoles) of the analogue (contained in 10 µl methanol) in a stoppered 1 ml glass vial, the following additions were made, in sequence, at room temperature, and with thorough mixing after each addition:-

1. 50 µl 0.1M phosphate buffer (pH 7.1; without preservatives)
2. 1 µg. lactoperoxidase (Sigma; 21.4 ml; enzyme activity checked:

ratio of O.D. at 412 nm to O.D. at 280 nm = 0.75; 1 µg. added in vol volume of 1 µl deionized water.

3. 2 mCi $Na^{125}I$ (Product IMS 30; Amersham; iodine in 20 µl carrier-free in NaOH solution, pH 7-11, free from reducing agent).

4. 40 ng hydrogen peroxide (Hopkins & Wilkins; spectrophometric verification of stock solution at 230 nm, molal absorptivity = 72.4; 40 ng added in 10 µl deionized water).

These reagents were mixed on a vortex mixer for 12 min and then 100 µl of 16% sucrose in a buffer of 0.022 M boric acid, 0.0064 M disodium EDTA and 0.014 M Tris (pH 8.9; all reagents from Sigma) were added. The products of iodination were separated by polyacrylamide gel electrophoresis. Glass rods (5 x 75 mm; Pharmacia) filled to 65 nm with 7.5% polyacrylamide were used for this separation. Each gel rod was loaded with 50 µl of the iodination and sucrose mixture. A continuous water-cooled buffer system of 0.011 M boric acid, 0.0032 M disodium EDTA and 0.007 M Tris (pH 8.9; all reagents from Sigma) was used and the gels were subjected to anodic electrophoresis at a constant current of 4mA/gel for between 60 and 90 minutes. On a separate gel, 50 µl of a bromophenol blue BSA solution (0.1% bromophenol blue, 1% BSA, 16% sucrose in buffer containing 0.011M boric acid, 0.0032 M disodium EDTA and 0.007 M Tris, pH 8.9-9.0) were run for calibration purposes (the bromophenol blue migrates as a broad diffuse band, in front of the BSA which is characterised by a narrow deep blue band). Electrophoresis was stopped when the BSA band had migrated approximately 2/3 through the gel. At this time the monoiodinated analogue would be expected to be 1/2-way, and the diiodinated analogue 2/3-way through the gel (Dr. M.D. Rollag, personal communication).

Following electrophoresis, the gels were extruded and sectioned into 17 segments, each segment being approximately 4 mm in width. Each segment was eluted for 12-16 hr (at 4°C) in 1 ml 0.01M phosphate buffer

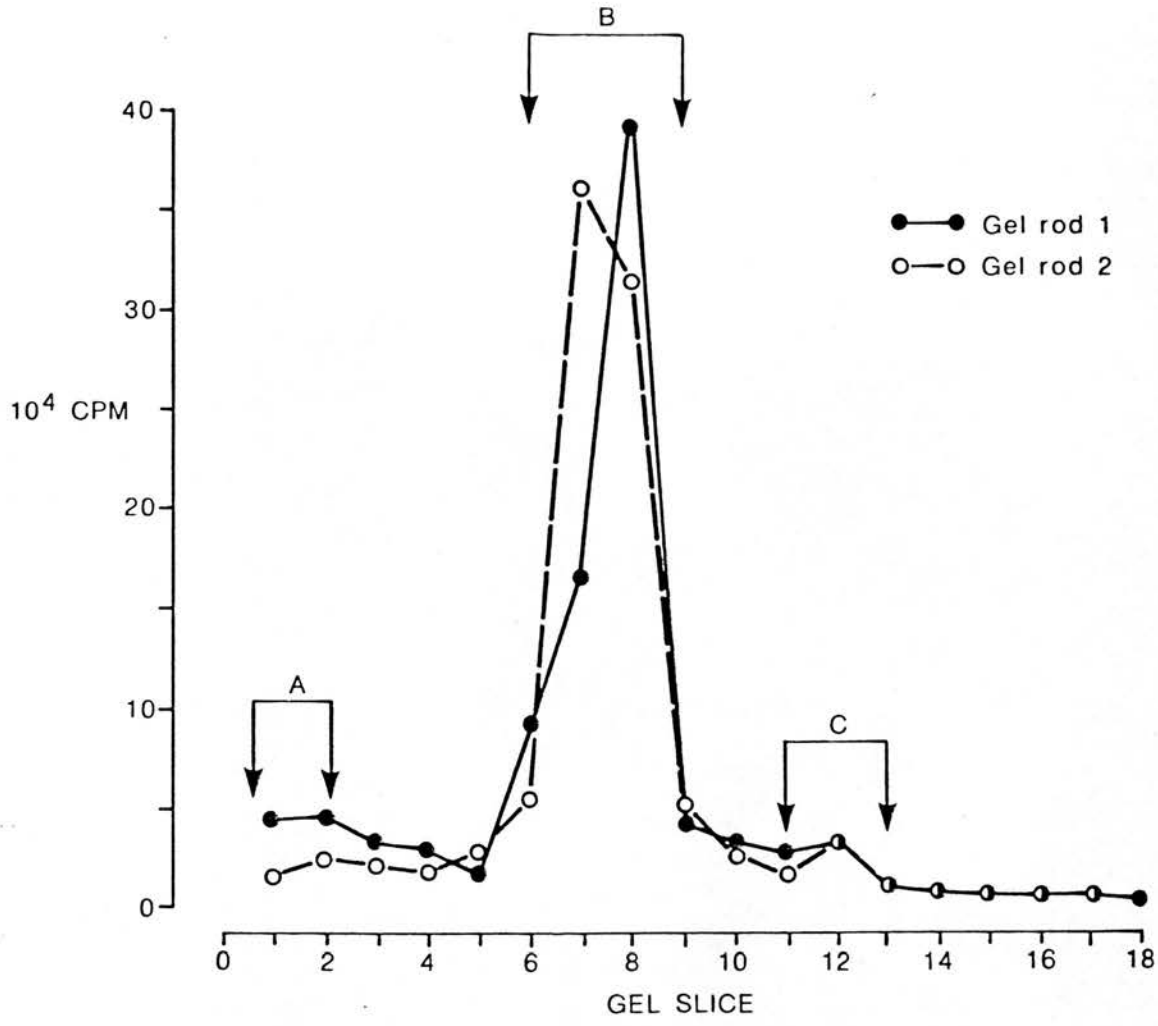


Fig. 2.3 A typical electrophoretogram (PAGE) obtained on separating the products of radioiodination of the MEL analogue.

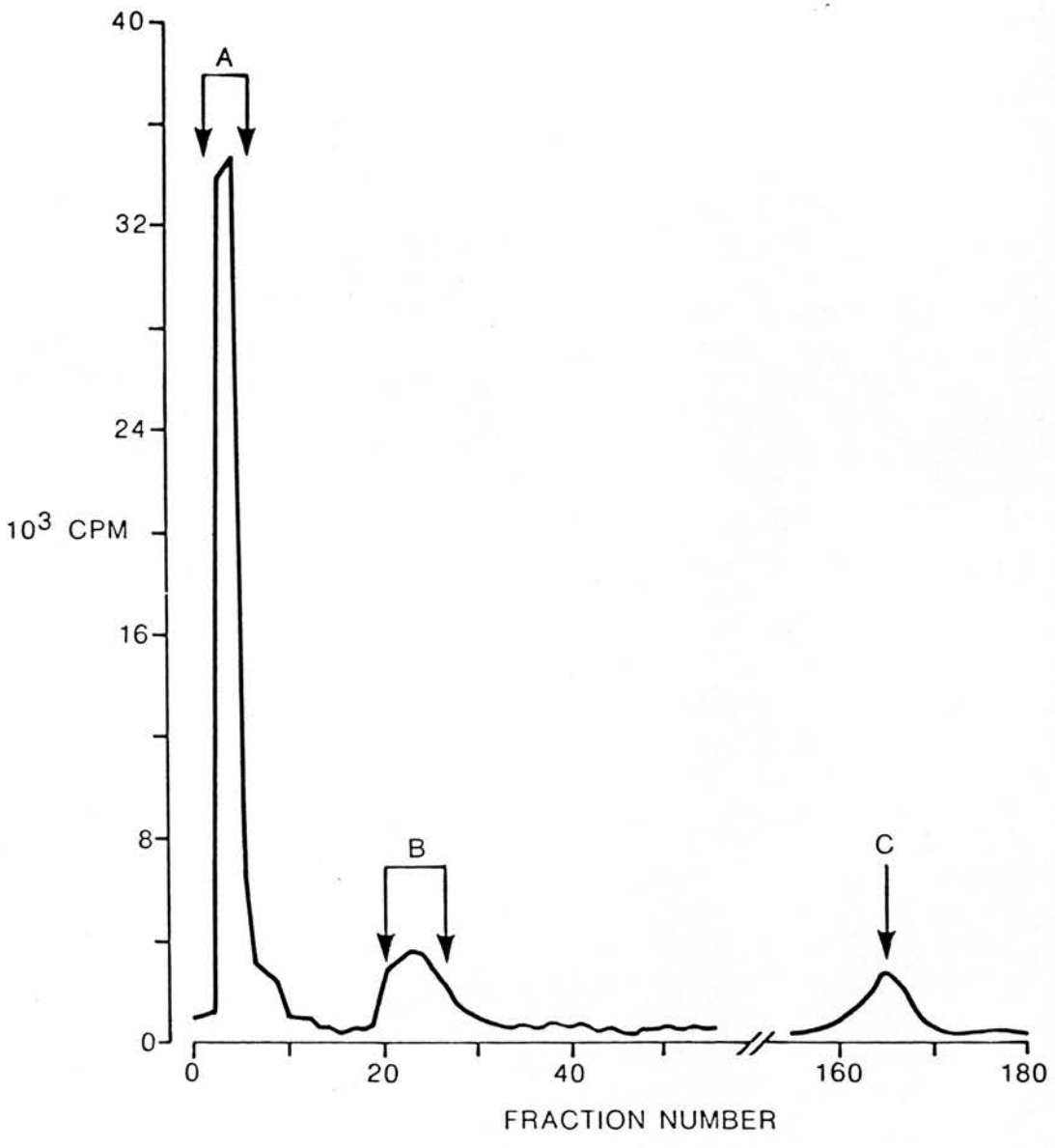


Fig. 2.4 Elution profile obtained from Sephadex G-25 chromatography column used to separate the products of radioiodination of the MEL analogue.

(containing 1% gelatin and 0.1% thiomersal, pH 7, all reagents supplied by BDH Chemicals). A typical separation profile is shown in Fig. 2.3. In every iodination performed (n=27), three peaks were obtained: the peak at the anodic end corresponded to the diiodo-form, that in the middle to the monoiodo-form and that at the cathodic end to unreacted iodine and other products of the iodination. Aliquots of the middle (monoiodo-form) peak were tested for specific binding in the RIA described in the following section. Those eluates which showed 20-40% specific binding were pooled and stored at 4°C. Aliquots from this pool were diluted in phosphate-gelatin buffer to give 40,000 - 50,000 cpm/100 ul, for use in the RIA (as it was not possible to quantify how much of the analogue was monoiodinated, the specific activity of the tracer could not be determined). The purity of the tracer and its possible deterioration with time, was not tested, but reproducible results were obtained with the same preparation for up to 6 weeks.

On 6 occasions, 100 µl 0.01M phosphate gelatin buffer was substituted for the 100 µl 16% sucrose in boric acid, EDTA and tris buffer in the above procedure. On these occasions the products of iodination were separated by gel chromatography using a 1x15 cm column containing Sephadex G-25 (Pharmacia) equilibrated with preservative-free 0.01M phosphate buffer (pH 7.1). Prior to loading, the packed gel was coated with a solution of 1% BSA in 0.01M phosphate buffer. The iodinated mixture (190 µl) was eluted with 0.01M phosphate buffer and collected in 2ml fractions. A typical elution profile from this method of separation is shown in Fig. 2.4. This method of separation was abandoned since the elution of the monoiodinated-analogue peak took between 4 and 8 hours, and because it had a relatively low specific binding (10-15%).

Radioimmunoassay of Melatonin

The extraction and assay procedures described are based on the

methods of Rollag & Niswender (1976) and Dr. M.D. Rollag (personal communication). All the reagents used were of analytical grade and obtained from BDH Chemicals unless specified.

Additions of plasma, standards and diluent buffers were made using either precision dispensing instruments (Gilson, Eppendorf) or an automatic dispensing system (Micromedic Systems Inc.) Additions of antiserum and labelled hormone were made using a 5.0 ml capacity, 0.1 ml graduated, Hamilton repeating syringe dispensor. Chloroform, petroleum ether and ethanol were dispensed using Oxford Repeating Dispensers. All determinations were made in duplicate.

(a) Extraction: Plasma samples, quality control samples, "blanks" (distilled water), and plasmas spiked with ^{3}H-Melatonin (New England Nuclear, Gm BSA; 36 Ci/mmol), were extracted in 12 x 75 mm glass tubes as follows:-

1. Add 200 µl sample
2. Add 500 µl 0.1M carbonate buffer, pH 10.25
3. Add 2 ml chloroform
4. Mix on multi-vortex shaker for 30 min, at 20°C
5. Remove upper aqueous phase by aspiration (after centrifuging if interphase is not well marked)
6. Add 1 ml distilled water
7. Mix on multi-vortex shaker for 5 min at 20°C
8. Centrifuge at 2000 g for 30 min at 4°C
9. Remove upper aqueous phase by aspiration
10. Dry organic phase at 40°C under nitrogen
11. Reconstitute in 500 µl 0.01 M phosphate buffer (with 0.9% NaCl, (pH7)
12. Mix and incubate in darkness for 16-24 h at 20°C
13. 2 ml petroleum ether (40°-60°C b.p.)
14. Mix on vortex mixer

15. Freeze aqueous layer in dry ice/ethanol bath; decant organic phase (waste).

16. Thaw out aqueous phase at 20°C in darkness.

The extraction efficiency of this method was checked by comparing the ^{3}H-MEL activity in the plasma samples spiked with ^{3}H, with that in unextracted plasma spiked with ^{3}H-MEL. Liquid scintillation counting was performed on a Packard 3600 instrument, using 0.4% butyl-PBD in toluene as the scintillation fluid. The mean efficiency of this extraction procedure was 82.2% (n=30), with a co-efficient of variation of 9.5%. Corrections for recovery were only made when the extraction efficiency for a particular assay was below 70%.

(b) Assay (modified from method of Rollag & Niswender, 1976): The assay buffer was 0.01 M phosphate buffer containing 0.9% NaCl 0.01% thiomersalate and 0.1% gelatin (pH 7.0).

The antiserum was used at an initial dilution of 1/64000 in 0.01M phosphate buffer containing 0.9% NaCl and 0.005% rabbit γ-globulin (Miles Laboratories). In the early versions of this assay 0.05 M EDTA was included in this buffer, but was later excluded as it was found to interfere in the assay in an unpredictable manner.

A stock standard solution containing 1 mg melatonin/ml ethanol was prepared and stored at -20°C. (The melatonin was obtained from Sigma). This solution was diluted, in 3 serial dilutions, to give a "working standard solution" of 1 pg melatonin/ml. These dilutions were made in the assay buffer. Fresh batches were prepared for each assay. Standards used in the assay ranged from 1 to 500 pg/tube (equivalent to 5-2500 pg/ml) and were made up to a volume of 500 µl with assay buffer.

Dilutions of the ^{125}I-melatonin analogue, calculated to provide 40,000-50,000 cpm/100 µl, were also made in the assay buffer.

The assay procedure consisted of the following steps: to each of the tubes containing standard solutions, 500 µl assay buffer, or plasma samples, 200 µl of the diluted antiserum were added (200 ul of 0.01 M phosphate buffer containing 0.9% NaCl and 0.005% normal rabbit γ-globulin were added to those tubes set up to determine "non-specific binding"). The contents of the tubes were mixed on a vortex mixer and then incubated for 24 h at 0-2°C. One hundred ul of diluted ^{125}I-melatonin analogue were then added to each tube, and the contents of the tubes were mixed and incubated for 48 h at 0-2°C. Following this incubation, 2 ml ethanol were added to all the tubes (except those used to monitor the total amount of radioactivity added), and the tubes were centrifuged at 2000 g for 30 min at 4°C. The antibody-bound and free ^{125}I-melatonin analogue fractions were separated by decanting the liquid contents of the tube. The ^{125}I-activity of the pellets (bound fraction) was determined in a γ-spectrometer (LKB).

(c) Calculation of results: The assay results were calculated using a programme written by Dr. R. M. Sharpe for a desk-top calculator (Hewlett-Packard 9821 A). In calculating specific binding, allowance was made for non-specific binding. A logit-transformation was performed on the binding data for the standards, and the method of least squares was used to fit a regression line through the data points. Complete details of the statistical procedures incorporated in the program are described in Sharpe (1979). The upper and lower "cut-off" points for interpolation were 90% (of zero-binding) and 10% (of zero-binding) respectively. Other calculations (e.g. correction for recovery subtraction of "extraction blanks" and conversion of values in terms of pg/tube to pg/ml) were peformed manually.

(d) Assay characteristics: A typical standard curve is shown in

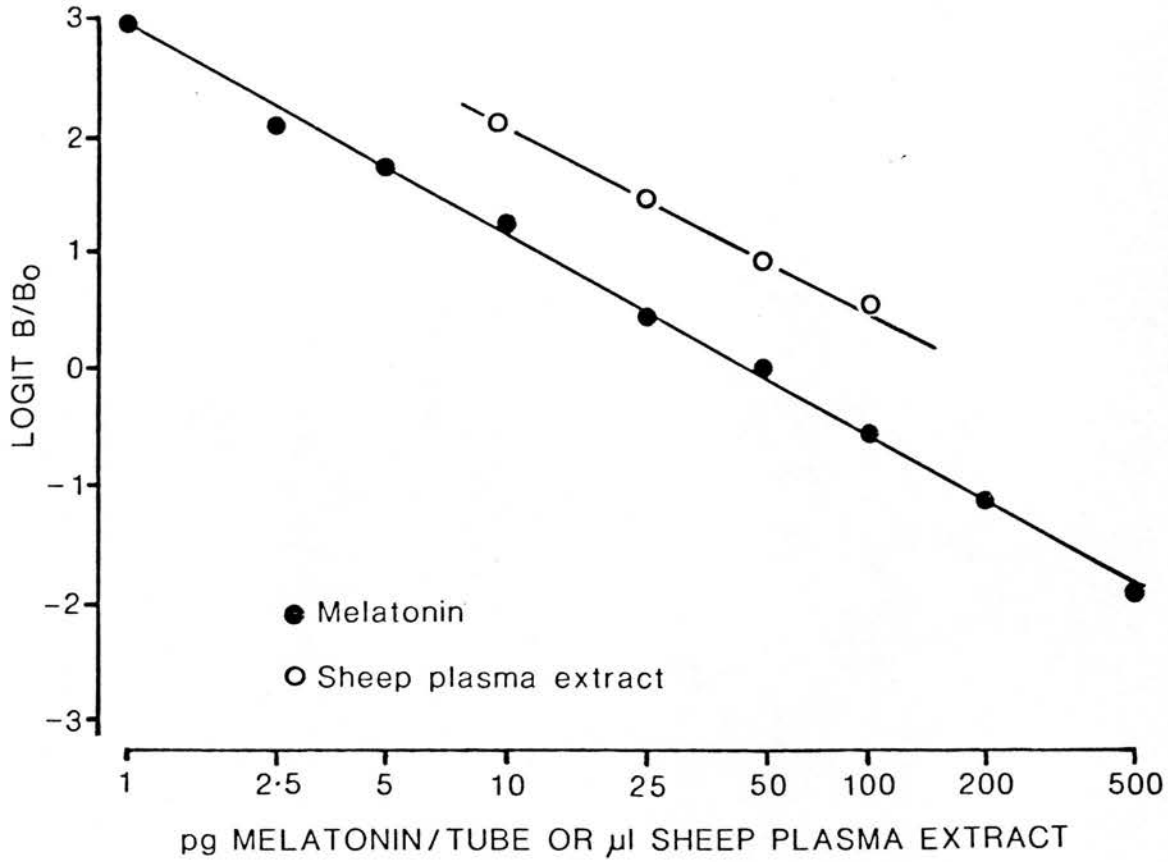


Fig. 2.5 Displacement (of ^{125}I-MEL analogue) curves obtained with varying amounts of MEL or extracts of ovine plasma.

Fig. 2.5 Fifty percent inhibition of ^{125}I-MEL analogue binding was achieved by 27 pg of MEL and the "least detectable dose" (90% cut-off point) was 3.5 pg/tube, in the illustrated example. Extraction blanks never exceeded 25 pg/tube.

Validation of MEL RIA

The RIA method described above has already been validated by Rollag and Niswender (1976) for use with ovine serum, but nevertheless, a number of validation tests were carried out in the laboratory since most of the samples were of blood plasma rather than of serum in the present study.

(a) Chromatographic identity: Three pools of ovine plasma (collected during the middle of the night, middle of the day, and one made from a mixture of day and night-time samples) were incubated with ^{3}H-Melatonin (New England Nuclear) for 3 hr at room temperature. After extraction of the samples, as described above, aliquots of the extracts were applied to Eastman No.6060 Chromatogram Sheets (Kodak) and chromatographed in chloroform: methanol (9:1). Each chromatogram was cut into 28 equal segments, and the segments were eluted in 1 ml assay buffer for 16 h at room temperature. The eluates were assayed by both liquid scintillation spectrometry and RIA.

As shown in Fig.2.6, ^{3}H-MEL co-migrated with immunoreactive MEL (Rf values: 0.46-0.68).

(b) Parallel inhibition: Fig. 2.5, showing a typical standard curve in the RIA, also shows the displacement of the ^{125}I-MEL analogue by various volumes (10,25,50 and 100 µl) of an ovine plasma extract. The slopes of the logit-transformed data for the standard curve and plasma extract displacement curve were -0.801 and -0.92 respectively: these slopes were found not to be significantly different.

(c) Accuracy: Varying amounts of MEL were added to aliquots of ovine

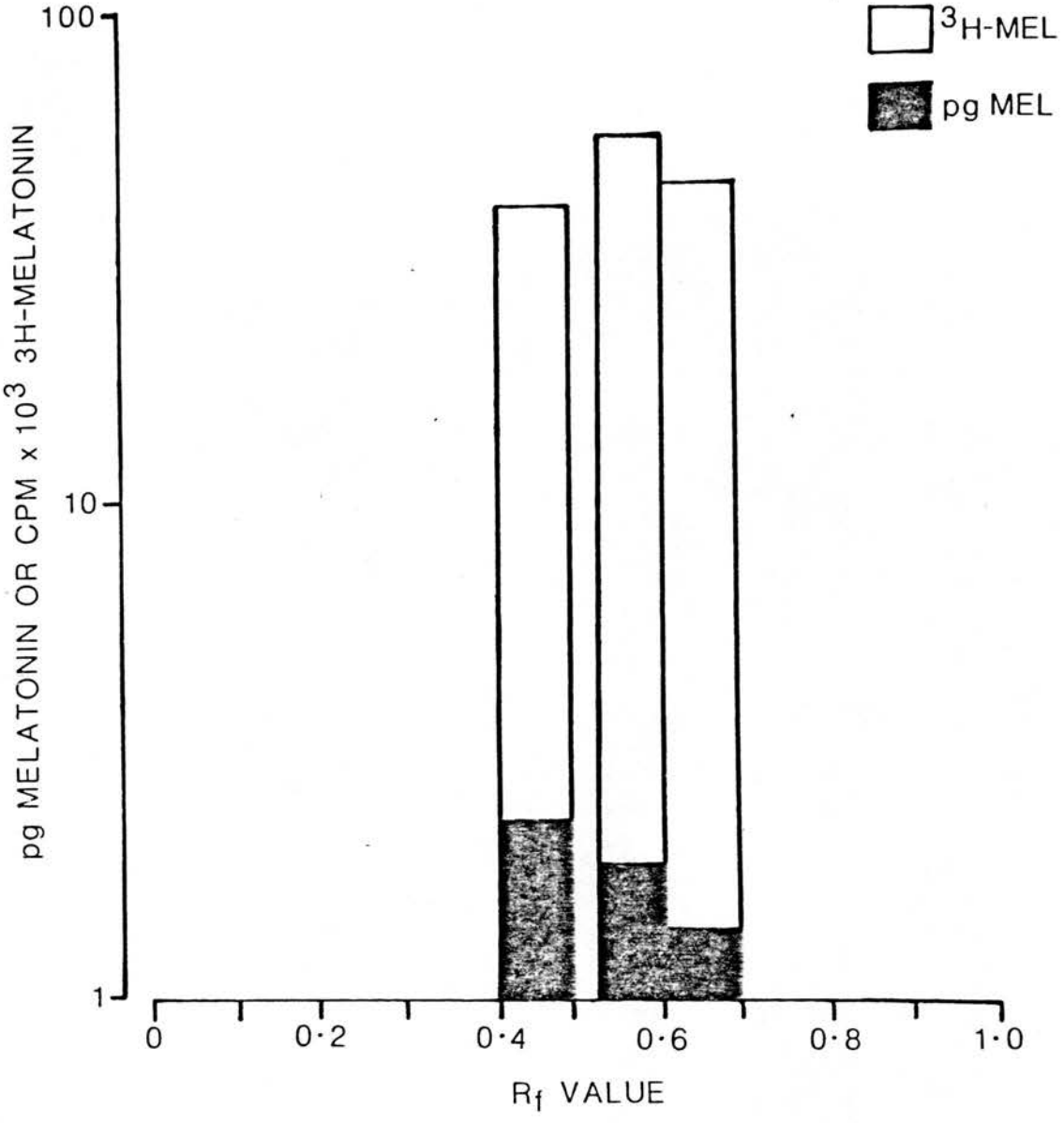


Fig. 2.6 Chromatographic localization of MEL immunoreactivity and ^{3}H-MEL (details in text).

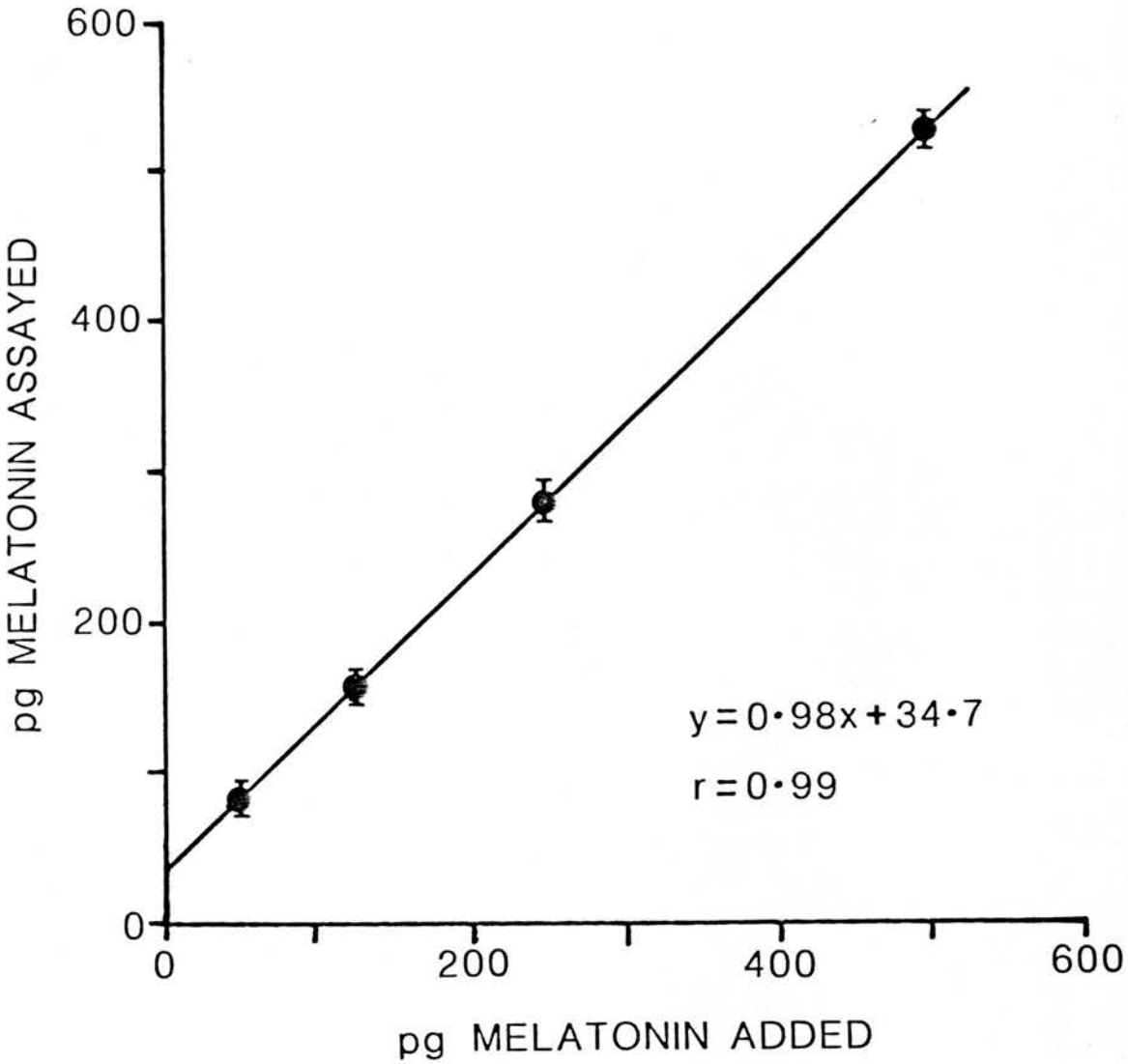


Fig. 2.7 Recovery of MEL added to daytime ovine plasma already containing 34.7 pg MEL/ml. Each point represents the mean (± S.E.) of 4 determinations.

plasma collected during day-time to give plasma concentrations of 50, 125, 250 and 500 pg/ml, and the various pools were extracted and assayed, in quadruplicate by RIA. The results of the test are shown in Fig. 2.7; there was a significant correlation between the assayed concentrations and the theoretical concentrations of the 4 plasma pools (the y-intercept of the fitted line in Fig. 2.7 indicates that the endogenous MEL content of the plasma was 34.7 pg/ml).

(d) Intra-assay variation: This parameter was assessed by assaying aliquots of 3 ovine plasma "quality control" pools in sextuplicate in the same assay. The coefficients of variation for the various pools were 21.4%, 12.3% and 9.7% at concentrations of approximately 30,75 and 130 pg/ml, respectively.

(e) Inter-assay variation : three quality control pools of ovine plasma were assayed, in duplicate, on 25 consecutive occasions. The inter- assay coefficients of variation were found to be 26.6%, 14.6% and 8.9% for plasma pools with mean MEL concentrations of 28.0, 77.4 and 127.4 pg/ml respectively.

(f) Plasma vs. Serum Extracts : Rollag & Niswender (1976) described their assay for use with blood serum samples. Most of the samples used in this study however, were of blood plasma. To test for possible differences in estimates obtained for the two fluids, plasma and serum were prepared from a single blood pool collected from a Soay ram during darkness. The plasma and serum were extracted identically and the MEL content of their extracts measured by RIA. The two fluids were found to contain nearly identical amounts of MEL.

2.5.2. Radioimmunoassay of ovine prolactin

A homologous, double-antibody RIA (Lamming, Moseley & McNeilly, 1974; McNeilly & Andrews 1974) was used to measure blood plasma

concentrations of prolactin. The lower limit of detection of the assay (90% of zero-binding) was 1.6 ng/ml. The intra- and inter-assay coefficients of variation were 4.3% and 12.2% respectively. The reference standard used was NIH-P-S9 obtained from the Pituitary Agency of the National Institute for Arthritis, Metabolic and Digestive Diseases (USA). Computation of results was according to the method described by Sharpe (1979). All samples from one animal were measured in duplicate in a single assay or in adjacent assays.

2.5.3 Radioimmunoassay of Testosterone

Blood plasma concentrations of testosterone were measured, after extraction with hexane-ether (Corker & Davidson, 1978) by the RIA method described for human plasma by Corker & Davidson (1978). The characteristics of the assay described by these authors were found to remain unchanged when sheep plasma samples were assayed (Lincoln, Peet & Cunningham, 1977). The hormone concentrations were calculated, after correction for recovery (Corker & Davidson, 1978), by the methods described by Sharpe (1979). All samples from one animal were measured in a single assay or in adjacent assays.

2.6. STATISTICAL ANALYSES

Standard parametric tests (e.g. correlation coefficients, paired and unpaired Student's 't' test, analysis of variance or 'F' test) were used according to the conditions stipulated, and formulae given by (Winer, 1971).

Non-parametric analyses (e.g. Sign test, Marn-Whitney 'U' test, Rank Spearman Correlation Test) were used according to the rules and methods described by Siegal (1956).

The hourly hormone data for individual animals was analysed for peaks using a combination of the non-parametric "Sign Test" and Grubbs-Type statistics for the detection of outliers (Tietjen & Moore, 1972). The method was developed in conjunction with Mrs. Pamela Warner and Dr G.A.

Lincoln. It consisted of the following steps: The value of the median hormone concentration for each 24 hour period was computed for the results from the individual rams. b) Each hourly value was assigned as being above, below or equal to the median value for the 24 hour period in which it occurred. c) The high values which represented significant outliers for the values for the entire 24 hours were determined as described by Tietjen & Moore (1972). A significant peak in the hormonal profile was then taken as any period with at least 4 consecutive hourly values above the 24 hour median; at least one of these values had to be a significant outlier.

Detection of peaks in the blood levels of electrolytes was not possible by the outlier method due to the gradual build-up and decline of concentrations during 24 hours. However, the cosistency of the results allowed the use of the Sign Test alone - a peak was defined as a period when at least 4 consecutive values were above the 24 hour median for the period in which they occurred.

An estimate of the duration of the rhythms in the concentrations of MEL, PRL and electrolytes was obtained by calculating the interval between the start of each significant peak, for each animal.

CHAPTER 3

PATTERNS OF MELATONIN IN THE BLOOD AND THE PHOTOPERIODIC RESPONSES OF INTACT AND SUPERIOR CERVICAL GANGLIONECTOMISED SOAY RAMS

3.1 INTRODUCTION

The secretion of LH, FSH and PRL, and the activity of the testes are known to be altered in rams subjected to changes in the natural or artificial daylength (LH, FSH and testes: Lincoln & Davidson, 1977; Lincoln, Peet & Cunningham, 1977; PRL: Pelletier, 1973; Ravault, 1976; Lincoln, McNeilly & Cameron, 1978). Information about the photoperiod is believed to reach the hypothalamo-pituitary unit of mammals via the pineal gland, since pinealectomy or sympathetic denervation of the gland abolishes the effects of daylength upon these various processes (Lincoln, 1979a, 1979b; Turek & Campbell, 1976; Lincoln & Short, 1980; Lincoln, Almeida, Klandorf & Cunningham, 1982; Seamark, Kennaway, Matthews, Fellenberg, Phillipou, Kotaras, McIntosh, Dunstan & Obst, 1981). Support for the idea that the pineal might play the role of photo-transducer comes from the fact that the gland secretes a variety of indoleamines in patterns which closely reflect the daily external LD cycle (Quay, 1974; Balemans, 1979).

The aim of the experiment to be described here was to discover (a) whether there are differences in the amplitudes or temporal patterns of blood concentrations of MEL in rams kept under long daily photoperiods (long days) and short daily photoperiods (short days), and (b) whether the removal of the innervation to the pineal by SCGx disrupts the patterns of MEL in the blood.

3.2 MATERIALS AND METHODS

Eight adult Soay rams held under alternating 16-week cycles of artificial long days (16L:8D) or short days (8L:16D) for more than 3 years were used in the study. The pineal glands of 4 of these rams had previously been denervated by surgical removal of the superior cervical ganglia (see 2.4). The remaining four animals (one of which had

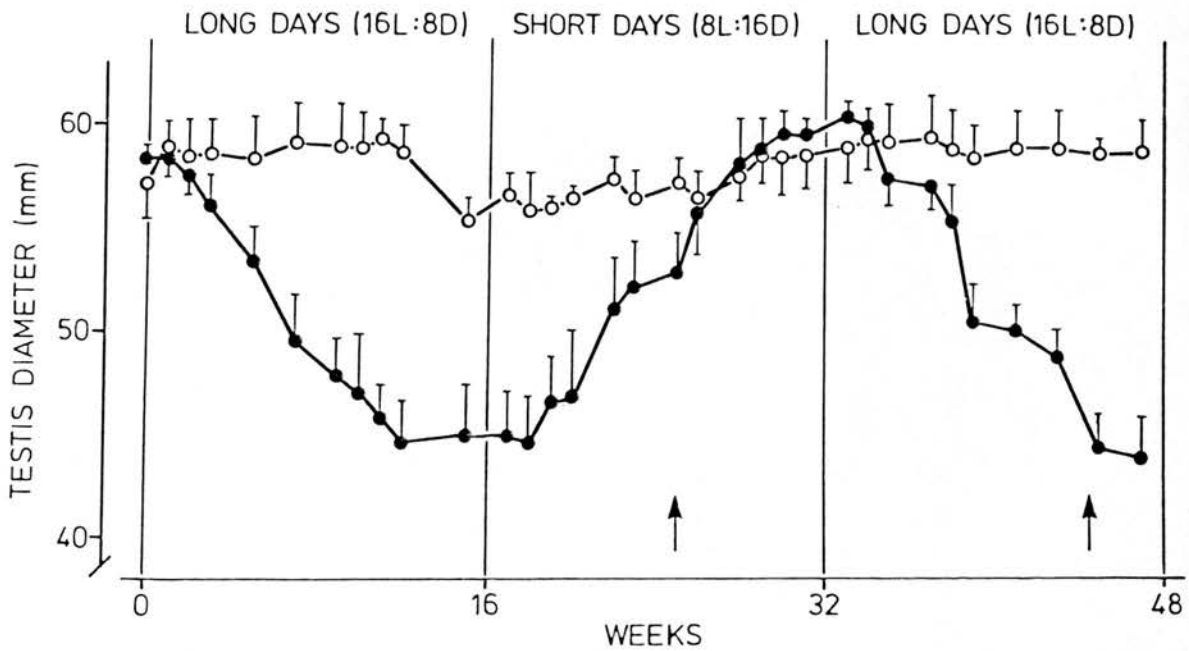


Fig. 3.1 Changes in the diameter of the testes (mean ± SEM) of 4 control (●) and 4 superior cervical ganglionectomised (SCGx; o) Soay rams during exposure to alternating 16 week periods of long days (16L:SD) and short days (8L: 16D). Hourly blood samples were collected for 28 hours on two occasions during the study at the times indicated (↑).

received the sham operation), served as controls.

At weekly intervals during the study, the diameters of the rams' testes were measured and recorded. On two occasions (once during a 16 week period of short days: 64 days since the change from long days to short days; once during a 16 weeks period of long days: 87 days since the change from short days to long days), jugular blood samples were collected via indwelling cannulae, at hourly intervals, for 24 h, from each of the rams. The blood was centrifuged within 1 h of collection and the plasma stored at -20°C until assayed for concentrations of MEL and PRL by RIA methods described in Chapter 2.

This experiment formed part of a larger study carried out in collaboration with Dr. G.A. Lincoln (Lincoln, Almeida, Klandorf & Cunningham, 1982).

3.3 RESULTS

3.3.1 Testicular Size

The size of the testes of the control rams varied in accordance with changes in the photoperiod (Fig. 3.1 and Table 3.1); the testes grew during exposure to short days and regressed during exposure to long days.

The testes of the SCGx rams remained large under both long days and short days, showing only small changes in diameter (difference between mean values under long days and short days: 1.2 mm, compared to 17.2 mm difference for control rams - See Table 3.1).

TABLE 3.1

Changes in the size of the testes and in the blood plasma concentrations of melatonin and prolactin (24h mean ± SEM) in 4 control and 4 SCGx soay rams maintained under alternating cycles of short days (8L:16D) and long days (16L:8D) each cycle lasting 16 weeks.

	GROUP	SHORT DAYS	LONG DAYS
Testicular diameter (mm)	Control	54.2 ± 1.4a	47.0 ± 1.0b
	SCGx	57.0 ± 1.2	58.2 ± 1.4
Melatonin	Control	125 ± 46	160 ± 27
	SCGx	67 ± 6	76 ± 16
Prolactin	Control	2.1 a,b	68.3 ± 14.9b
	SCGx	17.1 ± 3.5	16.3 ± 7.1

a = $P < 0.05$ compared to long days (Student's paired t-test)

b = $P < 0.05$ compared to SCGx group(Student's t-test)

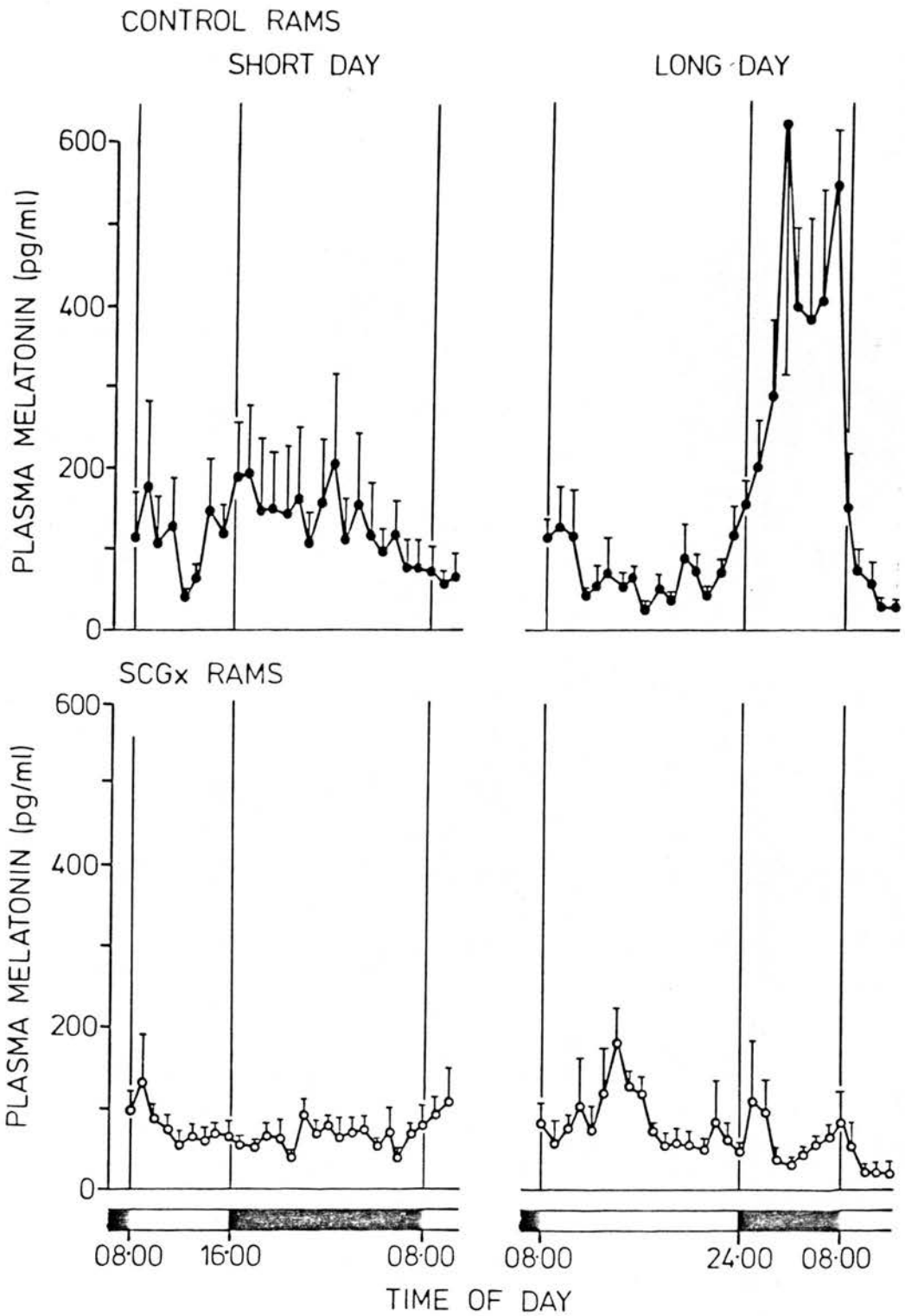

CONTROL RAMS
SHORT DAY
LONG DAY
PLASMA MELATONIN (pg/ml)
600
400
200
0
SCGx RAMS
PLASMA MELATONIN (pg/ml)
600
400
200
0
08·00
16·00
08·00
08·00
24·00
08·00
TIME OF DAY

Fig. 3.2 Changes in the blood plasma concentration of MEL (mean $\pm$ SEM) in 4 control (●) and 4 superior cervical ganglionectomised (SCGx; o) rams sampled hourly, for 28h, during short days and long days. The open boxes indicate the times of day over which "group peak" levels of MEL secretion occurred. The timing of the daily cycle of light and darkness is shown below.

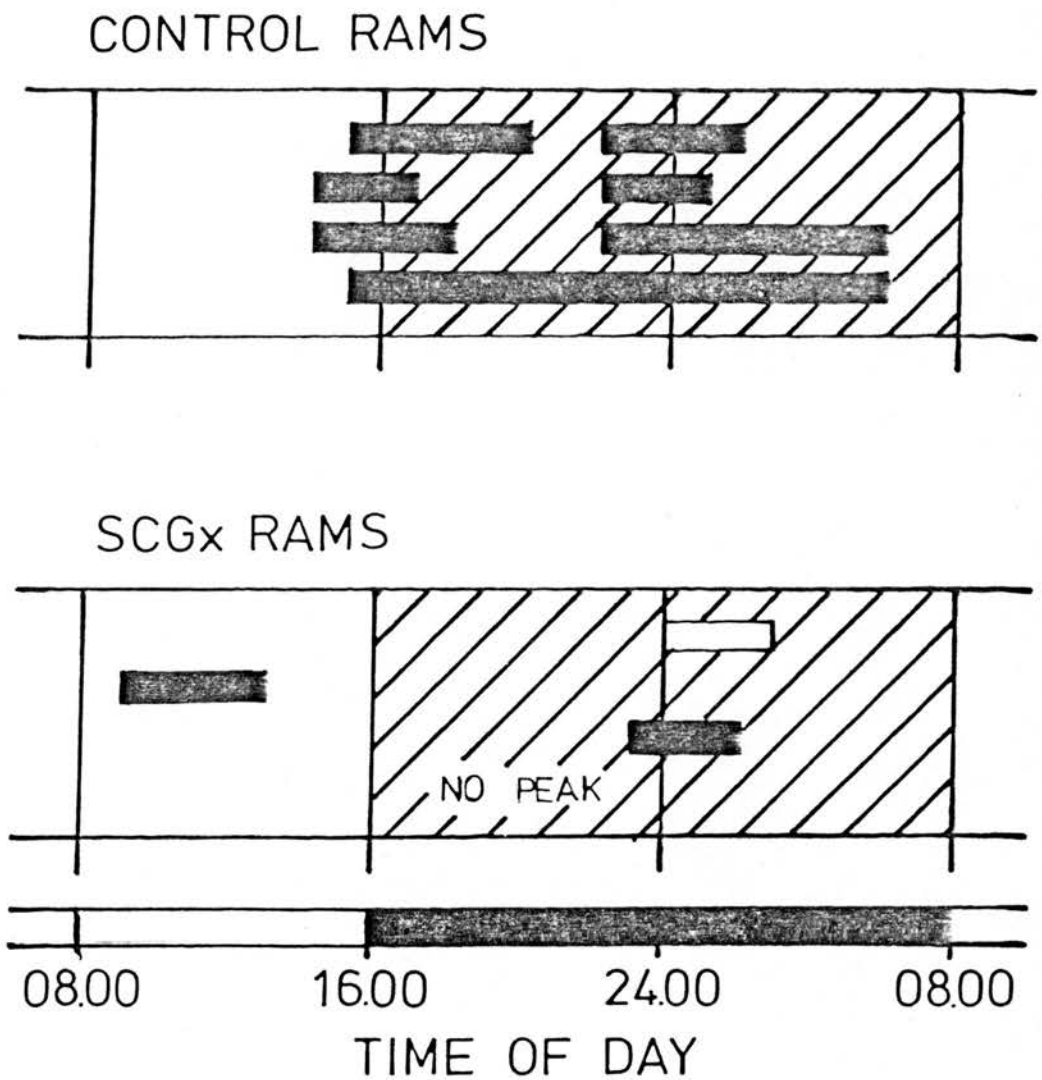


Fig. 3.3 Timing of the occurrence of peak MEL concentrations in the blood of 4 control and 4 SCGx Soay rams during a short day (8L:16D). Each bar in the same horizontal plane represents a peak for a single animal. (An open bar represents a period of high MEL levels which did not constitute a peak as defined in 2.6). The stippled areas represent periods of darkness.

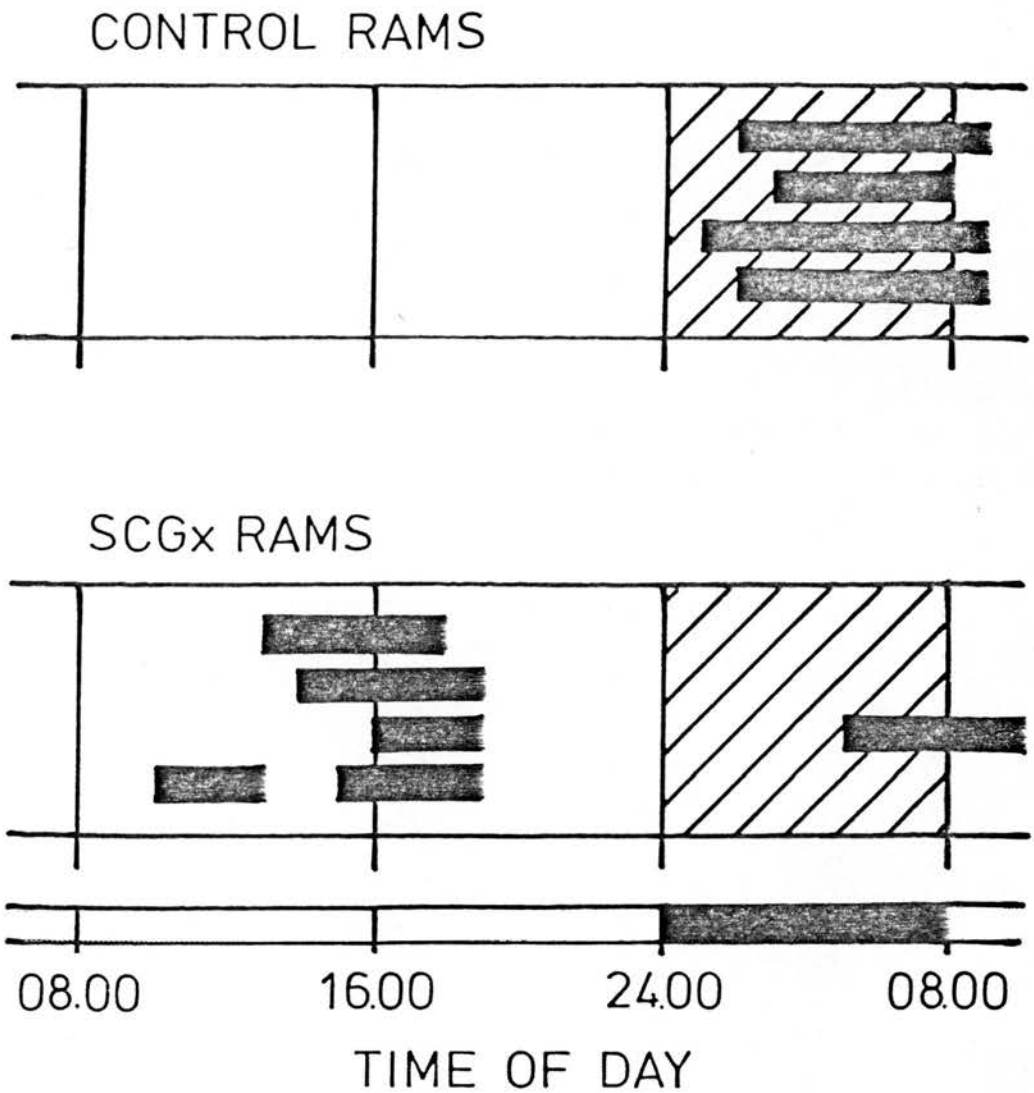


Fig. 3.4 Timing of the occurrence of peak MEL concentrations in the blood of 4 control and 4 SCGx Soay rams during a long day (16L:8D). Each bar in the same horizontal plane represents a peak for a single animal (some animals showed more than one peak). The stippled areas represent periods of darkness.

3.3.2 Melatonin

The control rams showed increased blood plasma levels of MEL during darkness under both short days and long days (Fig. 3.2, upper panel). The peaks occurred earlier in the day, corresponding to the earlier onset of darkness, in the short day group compared to the long day group (Figs. 3.3 & 3.4, upper panels). Under short days, peak blood levels of MEL occurred 1-2 hours in advance of the onset of darkness.

The night-time blood concentrations of MEL were significantly greater during long days than during short days (long days: 382 $\pm$ 52 pg/ml vs short days: 135 $\pm$ 10 pg/ml, $p < 0.05$, Student's paired 't' test). However, measurement of "areas under the curve" revealed no significant difference in the total amount of MEL found in the blood under long days and short days (long days: 5.2 $\pm$ 0.4 vs. short days: 4.8 $\pm$ 0.2 arbitrary units, $p > 0.1$, Student's paired 't' test). The 24h mean concentrations of MEL under long days and short days are compared in Table 3.1.

The SCGx rams did not show consistent night-time rises in their blood levels of MEL; indeed, on some occasions, small peaks of MEL were found during periods of light (Figs. 3.2, 3.3 and 3.4, lower panels). The mean daily concentrations and amounts of MEL in the blood of the SCGx rams did not differ under long days and short days (Fig. 3.2, Table 3.1). The 24h mean blood levels of MEL were significantly greater in the control rams than in the SCGx rams under both these photoperiods (Table 3.1).

3.3.3 Prolactin

The 24h mean blood plasma concentrations of PRL in the control rams were significantly lower during short days than during long days

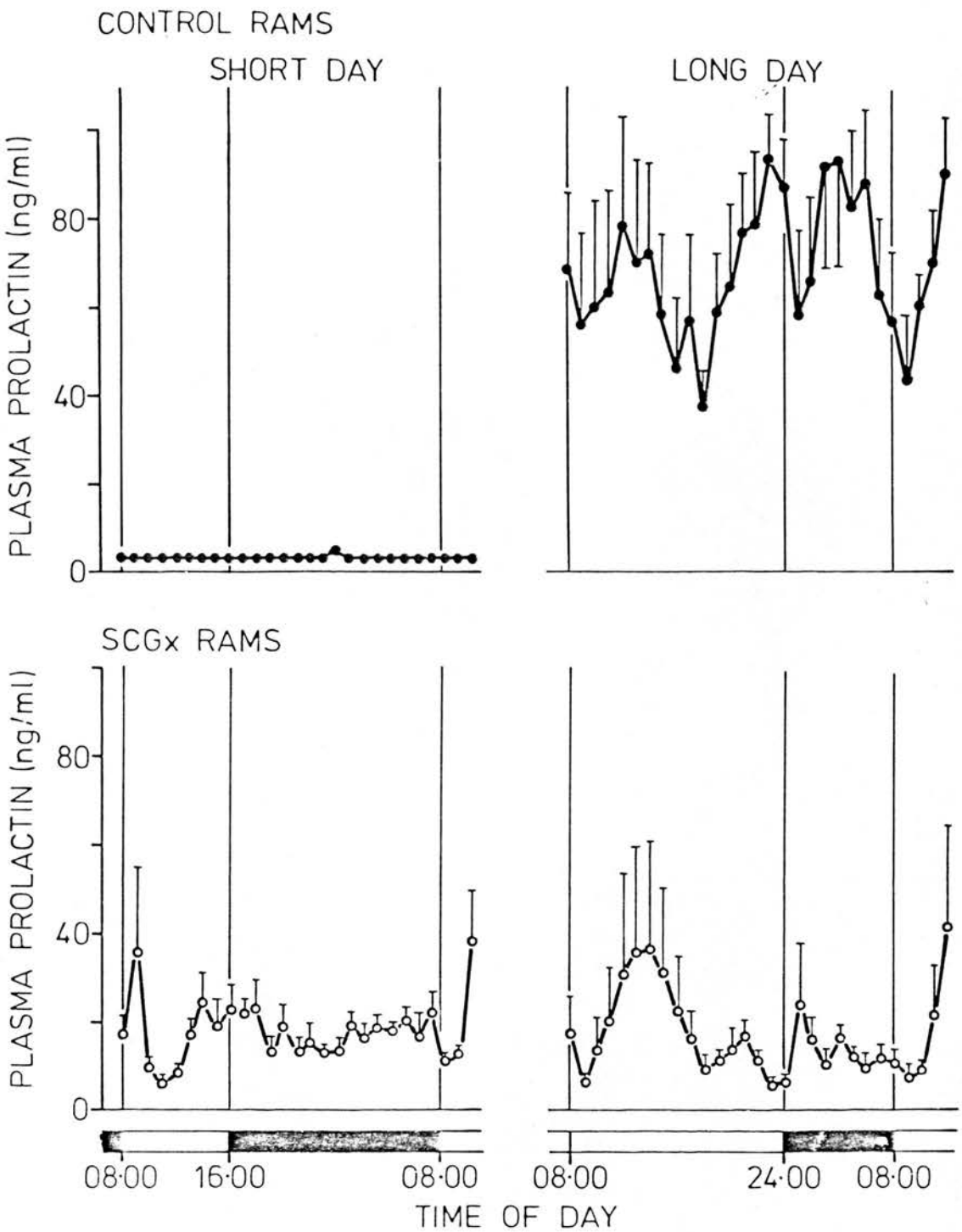

CONTROL RAMS
SHORT DAY
LONG DAY
PLASMA PROLACTIN (ng/ml)
80
40
0
SCGx RAMS
PLASMA PROLACTIN (ng/ml)
80
40
0
08·00
16·00
08·00
08·00
24·00
08·00
TIME OF DAY

Fig. 3.5 Changes in the blood plasma levels of PRL (mean $\pm$ SEM) in 4 control (●) and 4 superior cervical ganglionectomised (SCGx; o) rams sampled hourly, for 28h, during short days and long days. The timing of the daily cycle of light and darkness is shown below.

(Fig. 3.5, upper panel, Table 3.1), whereas no differences were found in the SCGx rams exposed to those two photoperiods (Fig. 3.5, lower panel, Table 3.1). Under short days, the SCGx rams showed significantly higher blood concentrations of PRL compared with the control rams; the reverse pattern was found during long days.

3.4 DISCUSSION

The blood plasma concentrations of MEL in the intact Soay rams were found to change consistently and in close relation to the external LD cycle. The highest levels were found mainly during periods of darkness, thus supporting data previously reported for the sheep (Rollag & Niswender, 1976; Kennaway et al, 1977; Arendt, 1979).

While the absolute amounts of MEL found in the blood were similar under long days and short days, the patterns of changes in the blood levels of MEL differed markedly under the two photoperiodic regimens. During short days, the peak blood concentrations of MEL occurred earlier in the day and for a greater number of hours than during long days; however, the amplitudes of these peaks were significantly smaller than those observed during long days. These data for the Soay ram differ from those obtained in a different breed of sheep, in one respect. While different patterns of MEL were found in the blood of Corriedale crossbred Western Range ewes at different times of the year, no seasonal differences were observed in the amplitudes of these levels (Rollag, O'Callaghan & Niswender, 1978).

In contrast to the findings of Rollag & Niswender (1976), a simple relationship between the patterns of appearance of MEL in the blood and the onset of light or darkness was not found in the present studies on the Soay breed. Those authors reported that the blood levels of MEL in sheep did not increase until the onset of darkness, and that the onset of light caused an immediate reduction in these levels. In this study, the control rams appeared to anticipate the onset of darkness

by showing large increases in the blood levels of MEL 1 - 2 h before "lights-out", while in several instances, MEL was still detectable in large concentrations in the blood 1 - 2 h after "lights-on".

MEL was found to be present in the blood of the SCGx rams, albeit in lower concentrations than those found in intact rams. While the SCGx rams showed fluctuations in their blood levels of MEL throughout the day, these fluctuations were not consistent between animals and they did not bear any relationship to the external LD cycle; peak blood concentratios of MEL occurred in individual SCGx rams at different times of the day even during periods of illumination. There was no discernible difference between the patterns of MEL in the blood of SCGx rams exposed to long days or short days.

Rams show marked reproductive responses to changes in the photoperiod; they grow their testes during short days and regress them during long days. Changes in the photoperiod also result in marked differences in the blood levels of PRL; low circulating levels of this hormone are found during short days while high levels are found during long days. The present data therefore suggest a correlation between the patterns of MEL in the blood and the photoperiodic responses of rams. The lack of photoperiodic responses, and of consistent LD-related changes in the blood levels of MEL in the SCGx rams lend support to this idea.

There are already several lines of evidence that MEL is involved in the photoperiodic control of ovine reproduction. Silastic implants of MEL were found to delay the development of the testes of Soay rams exposed to short daylengths, (Lincoln & Almeida, 1982), while the feeding of MEL to anoestrous Merino ewes led to the resumption of

ovarian activity during naturally long daylengths (D.J. Kennaway & R.F. Seamark, personal communication). Furthermore, when PINX Suffolk ewes held under long daily photoperiods (16L:SD) were infused with MEL according to a short day pattern (infusions made for 16 consecutive hours daily), reproductive activity was induced (E.L. Bittman, personal communication). Bittman's finding points to the importance of the temporal pattern of MEL availability in determining the reproductive response of sheep. Earlier studies in the golden hamster also noted that the timing of MEL injections was critical in determining the effects of a particular photoperiod, or indeed the hormone itself, upon reproduction (Tamarkin, Westrom, Hamill & Goldman, 1976; Tamarkin, Lefebre, Hollister & Goldman, 1977). The present data also suggest that MEL may act to inhibit reproduction, since the blood levels of MEL in the intact rams were greater during exposure to long days when the testes were regressed than during exposure to short days, when the testes were large. Also, the SCGx rams had depressed blood MEL levles while their testes remained active.

CHAPTER 4

TESTICULAR CHANGES AND PATTERNS OF MELATONIN AND PROLACTIN SECRETION IN SOAY RAMS EXPOSED TO LONG OR SHORT DAILY PHOTOPERIODS FOR PROLONGED PERIODS

4.1 INTRODUCTION

Alterations in the natural or artificial photoperiod may at first appear to simply induce or terminate reproductive activity. However, if animals are maintained under either a gonado-stimulatory or gonado-inhibitory photoperiod for an extended period of time, they may become insensitive, or refractory, to the normal effects of that photoperiod (see 1.3.4).

There are only a few reports on the effects of maintaining sheep under constant LD cycles for prolonged periods (e.g. Hampshire & Suffolk ewes: Cole, 1953; Merino ewes: Radford, 1961; Ille-de-France ewes: Thibault, Courot, Martinet, Mauleon, du Mesnil du Buisson, Ortavant, Pelletier & Signoret, 1966; Southdown ewes: Thwaites, 1965; Clun Forest ewes: Ducker, Bowman & Temple, 1973; Suffolk & Swaledale Border Leicester rams: Howles, Webster & Haynes, 1980). In all these studies, cycles of gonadal activity were observed, despite the prolonged exposure of the sheep to stimulatory or non-stimulatory daylengths, i.e. the normal effects of the photoperiod were ignored. Ducker et al (1973) and Howles et al (1980) suggested that sheep might possess an inherent rhythm of reproductive activity which becomes manifest in the absence of adequate changes in daylength. In addition, Howles et al (1980) concurred with the earlier suggestion of Lincoln & Davidson (1977) that the role of the photoperiod may be to entrain the time of the sexual cycle, rather than to cause it.

The involvement of the pineal, and in particular of MEL, in mammalian photorefractoriness has been reviewed earlier (1.4). Studies in the golden and Djungarian hamsters, the white-footed mouse and the ferret suggest that the photorefractory phenomenon might be accounted for by changes in (1) pineal activity or (2) the sensitivity of target

tissues to the effects of MEL (golden hamster: Turek, Desjardins & Menaker, 1976; Bittman, 1978a; Turek & Pappas, 1980; Hoffmann, 1981a-c; Stetson & Tate-Ostroff, 1981; Djungarian hamster: Hoffmann, 1981a-c; white-footed mouse: Johnston & Zucker, 1980a; ferret: Thorpe & Herbert, 1976; Herbert, 1981). These hypotheses stem from studies involving PINX or MEL supplementation. The one study in which the MEL content of the pineal was directly measured indicated that photorefractoriness in the golden hamster is not accompanied by changes in pineal MEL synthesis activity (Rollag, Panke & Reiter, 1980a).

The present study was intended to reveal any correlations which might exist between changes in testicular activity and the patterns of MEL in the blood of Soay rams exposed to either long days (16L:8D) or short days (8L:16D) for a period of nearly 2 years. Blood concentrations of PRL were also assayed for an additional indication of the rams' responsiveness to the two photoperiods.

4.2 MATERIALS AND METHODS

Two groups of 8 adult Soay rams were housed in separate, but adjacent, light-proof rooms. The first group ('L group') of rams had been exposed to a 16 week period of short daily photoperiods (8L:16D) prior to the start of the experiment (week 0) when they were transferred to a prolonged period (94 weeks) of long daily photoperiods (16L:8D). The second group ('S group') of rams had been exposed to a 16 week period of long days (16L:8D) before being transferred to an extended period (94 weeks) of short days (8L:16D).

Both groups of rams were maintained identically (as described in 2.1) and experienced the same daily fluctuations in ambient temperature.

At frequent intervals throughout the study, the diameters of the

testes of all the rams were measured using calipers (2.2). At weekly intervals, starting from week 17, a single 5 ml jugular-blood sample was collected from each ram by venepuncture (2.3); these samples were collected between 0800 and 1000h. On 15 occasions during the study (weeks 0,17,21,25,29,33,40,46,55,63,68,74,81,86 and 93), a cannula was inserted into the jugular vein of each ram and blood samples were collected at hourly intervals for periods of 25-30 hours. The weekly blood plasma samples were assayed for their concentrations of prolactin (PRL) and testosterone by the RIA methods described earlier (PRL: 2.5.2; testosterone: 2.5.3). Due to the constraints of time, the MEL concentrations in the blood plasmas of only 4 rams from each of the groups were assayed. MEL was measured by the RIA method described in 2.5.1. All the samples from a single animal were assayed together, in a single assay (PRL) or in adjacent assays (testosterone and MEL), to reduce the effects of inter-assay variation. The data were analysed by the various statistical procedures described in 2.6. Periods quoted for testicular regression or recrudescence to occur refer to the time interval between the attainment of minimum and maximum testis size.

At week 53 of the study one ram had to be culled due to his development of urethral calculi.

This study was carried out in collaboration with Dr. G.A. Lincoln, and technical assistance was provided by Norah Anderson (care of animals and collection of blood samples), Rhona Cunningham (serial bleeds and testosterone RIA) and Lorna Lambert (serial bleeds and PRL RIA).

4.3 RESULTS

4.3.1 Testicular changes

(a) 'L group': Throughout the study, the rams in this group showed

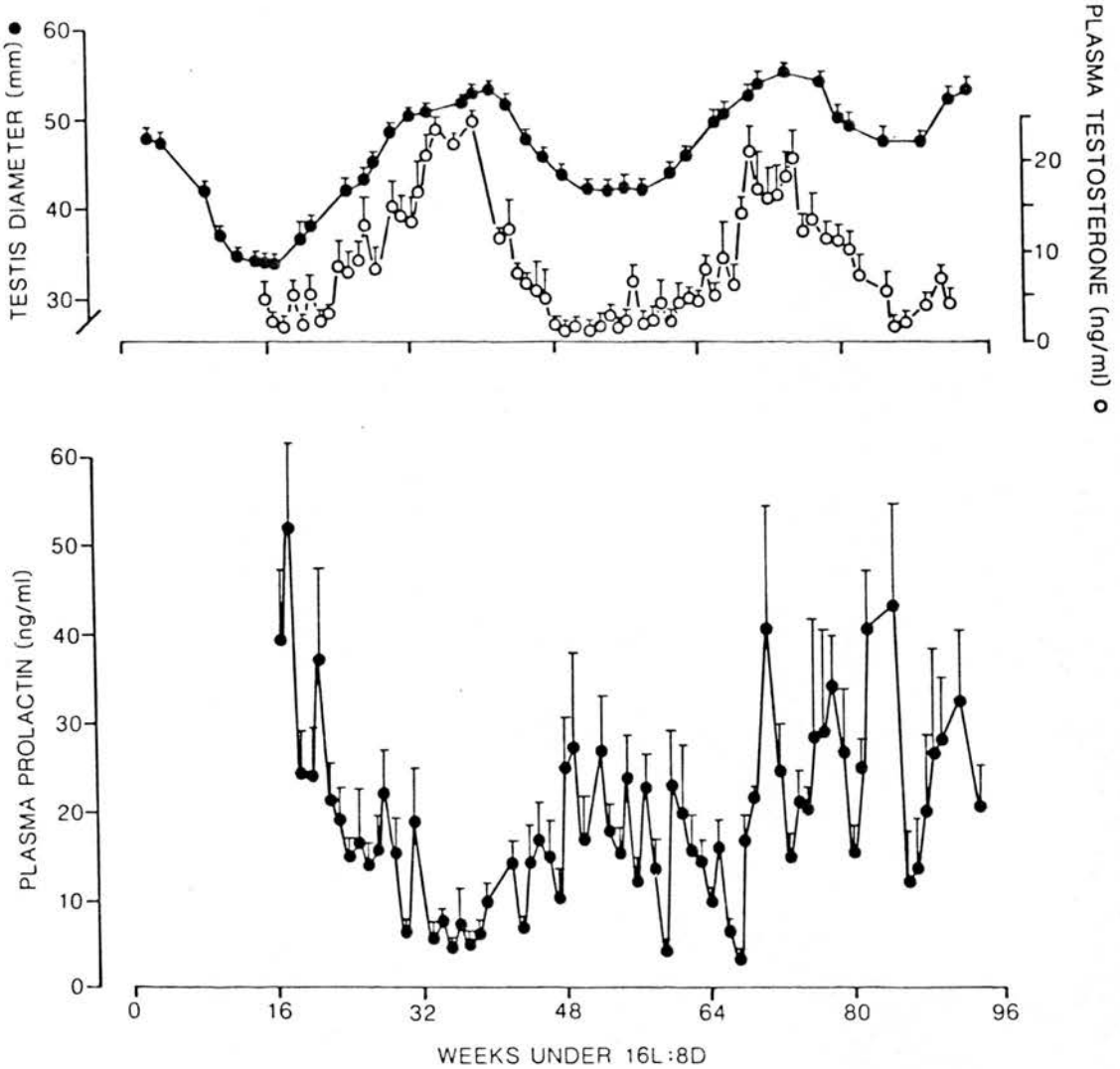


Fig. 4.1 Changes in testicular diameter and blood plasma testosterone concentrations, and blood levels of prolactin in adult Soay rams exposed to long daily photoperiods (16L:8D) for 94 weeks, having previously been exposed to 16 weeks of short daily photoperiods (8L:16D). Means $\pm$ S.E.M. are shown, and values are for 8 animals until week 53 when one of the rams was killed.

consistent changes in testicular activity (Fig. 4.1). Initially, during exposure to long days, all the rams regressed their testes (15.0 ± S.E.M. 0.4 weeks from the switch from short days to long days). Testicular growth then recurred, taking 25.3 ± 0.9 weeks to reach maximum size, after which alternating periods of testicular regression and recrudescence were observed until the end of the study (regression phase: 14.6 ± 1.7 weeks, followed by growth phase: 12.1 ± 2.6 weeks, and regression phase: 12.7 ± 1.1 weeks). The degree of testicular regression varied significantly (Student's paired 't' test, $p < 0.001$) on each occasion; 1st regression: 33.6 ± 0.5 mm, 2nd regression: 41.5 ± 0.9 mm, 3rd regression 47.0 ± 1.0 mm (Table 4.1). On the two occasions when testicular recrudescence was observed, however, the maximum sizes of the testes did not differ significantly (53.1 ± 0.3 mm and 54.9 ± 0.5 mm). The changes in testicular size were accompanied by parallel changes in the blood plasma concentrations of testosterone.

(b) 'S group': Consistent changes in testicular activity were also observed in this group of rams (Fig. 4.2). Initially, testicular growth was induced during exposure to short days (14.3 ± 0.8 S.E.M. weeks from the switch from long days to short days). This was followed by a phase of testicular regression (time for regression: 14 ± 0.5 weeks) and another phase of growth (time for maximum growth: 23.3 ± 1.6 weeks). Between weeks 52 and 94 of the study, cycles in testicular size were difficult to detect since the diameters of the testes varied only some 5 mm from the mean maximum diameter (Table 4.1). There was a significant correlation between testis size and blood plasma testosterone concentrations between weeks 17 and 52 of the experiment ($p < 0.001$);

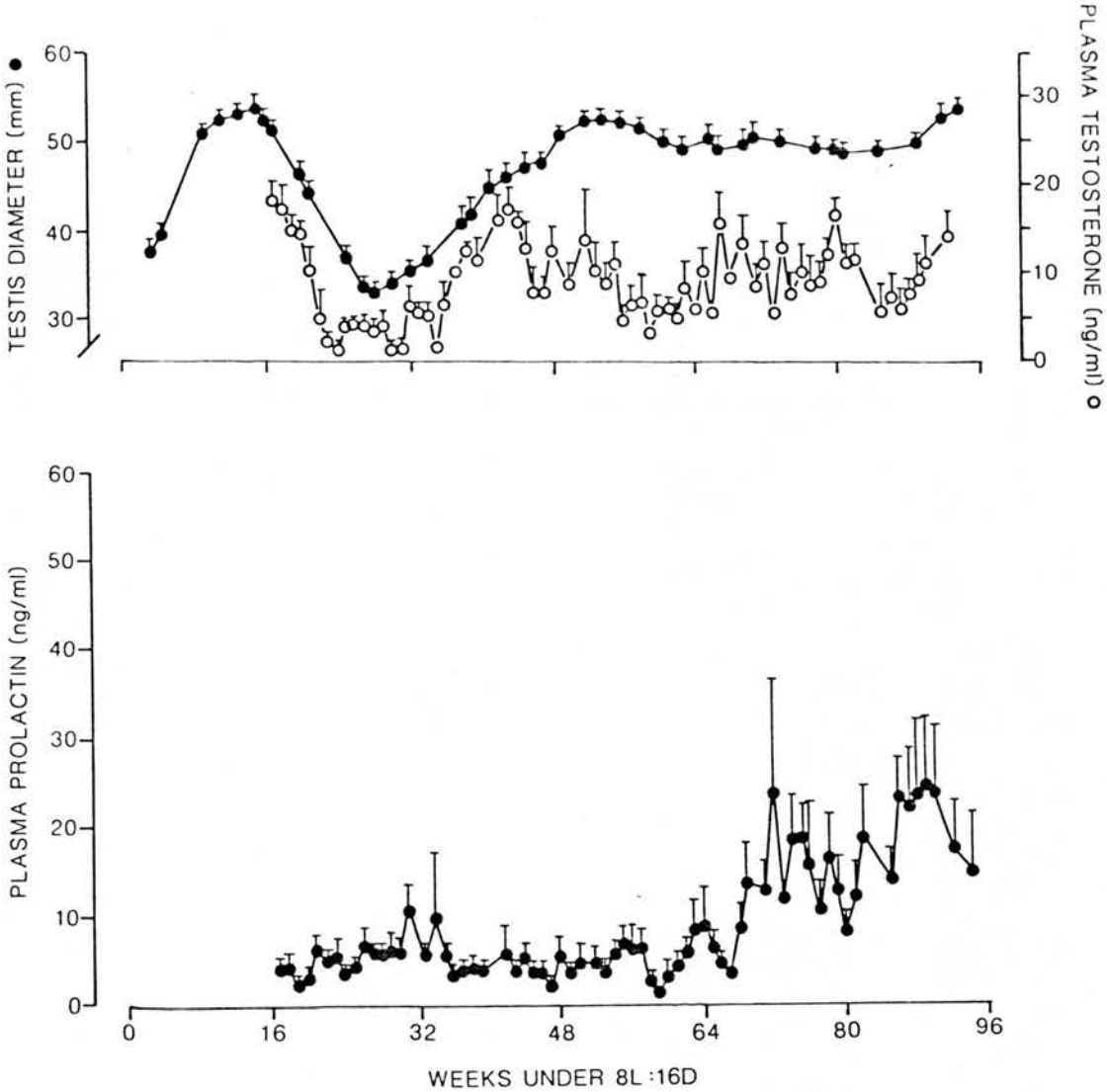


Fig. 4.2 Changes in testicular diameter and blood plasma testosterone concentrations, and blood levels of prolactin in adult Soay rams exposed to short daily photoperiods (8L:6D) for 94 weeks, having previously been exposed to 16 weeks of long days (16L:8D). Means $\pm$ S.E.M. are shown; n = 8.

thereafter, the plasma levels of testosterone continued to wax and wane despite the fact that the testes were maintained at about maximum diameter until the end of the study.

4.3.2 Changes in blood PRL concentrations

(a) 'L group': The long-term changes in the blood plasma concentrations of PRL are shown in Fig. 4.1. Until week 70, there was a significant inverse relationship between the blood plasma levels of PRL and testicular size ($p < 0.01$). Thereafter, a greater degree of variation was observed between the individual rams, and there was no longer a clear relationship between the blood levels of PRL and testicular activity.

(b) 'S group': PRL levels in the blood plasma of this group of rams remained low (< 15 ng/ml) between weeks 17 and 70, but increased to an average of 25 ng/ml for the remainder of the study (Fig. 4.2). At all times, the blood PRL levels of this group were significantly lower than those of the 'L group' ($p < 0.001$). There was no correlation between the changes in blood PRL concentrations and the changes in the testicular activity of these rams.

4.3.3 Changes in blood MEL concentrations

(a) 'L group': The hourly fluctuations in the mean blood levels of MEL on 6 occasions during the exposure of rams to a prolonged period of long days are shown in Fig. 4.3. With continued exposure to long days, there was an increase in the 24h mean blood levels of MEL; however, there was no correlation between the 24h mean concentrations of MEL and the long-term changes in the activity of the testes.

The daily profiles of the blood MEL concentrations were

TABLE 4.1

Summary of minimum and maximum testicular diameters found during successive periods of testicular regression and recrudescence in two groups of adult soay rams exposed to a prolonged period (94 weeks) of either long daily photoperiods ("L group": n = 8) or short daily photoperiods ("S group": n = 8).

TREATMENT GROUP	REGRESSION NO.	WEEK NO.	TESTICULAR DIAMETER (mm)*	
			MINIMUM (mean ± SEM) (n = 8)	MAXIMUM (mean ± SEM) (n = 8)
L	1	16	33.6 ± 0.5	
	2	54	41.5 ± 0.9	
	3	89	47.0 ± 1.0	54.1 ± 0.39
S	1	0	32.3 ± 0.7	
	2	28	32.8 ± 0.8	
	3	63	49.0 ± 1.2	
	4	81	48.6 ± 0.8	52.3 ± 0.59

* Only minimum testicular diameters differed significantly between cycles. Maximum testicular diameters were similar on successive phases of recrudescence, and the means for 3 such phases are shown.

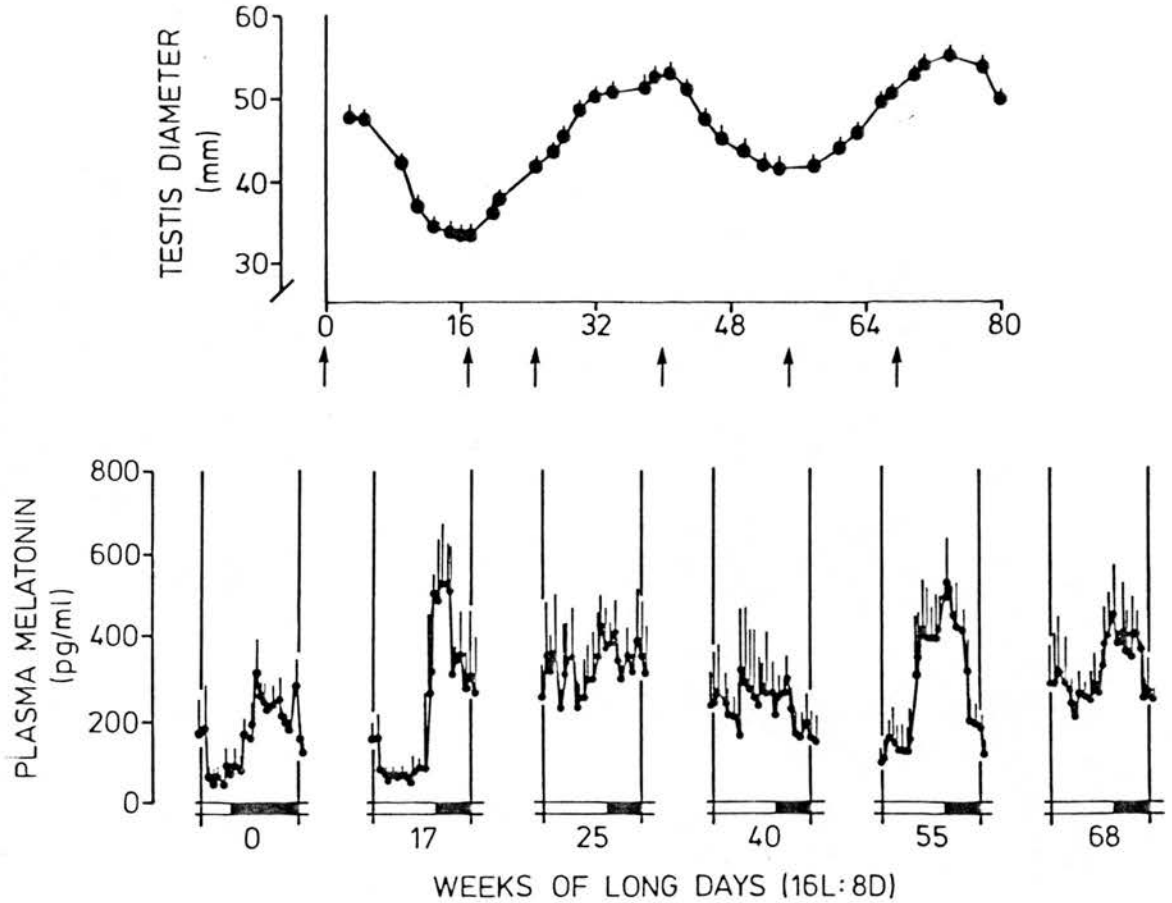


Fig. 4.3 Changes (mean ± SEM) in testis size and in the 24h patterns of melatonin concentrations in the blood plasma of 4 adult Soay rams exposed to a prolonged period of long daily photoperiods (16L:8D). Before transfer to the 16L:8D regime, the rams had been exposed to 16 weeks of short daily photoperiods (8L:16D). The data for week 0 is for the last day of that 16 week period of 8L:16D. The arrow-heads refer to the points at which the hourly blood collections were made (weeks 0, 17, 25, 40, 55 and 68). The timing of periods of light (open bars) and darkness (closed bars) are shown at the bottom.

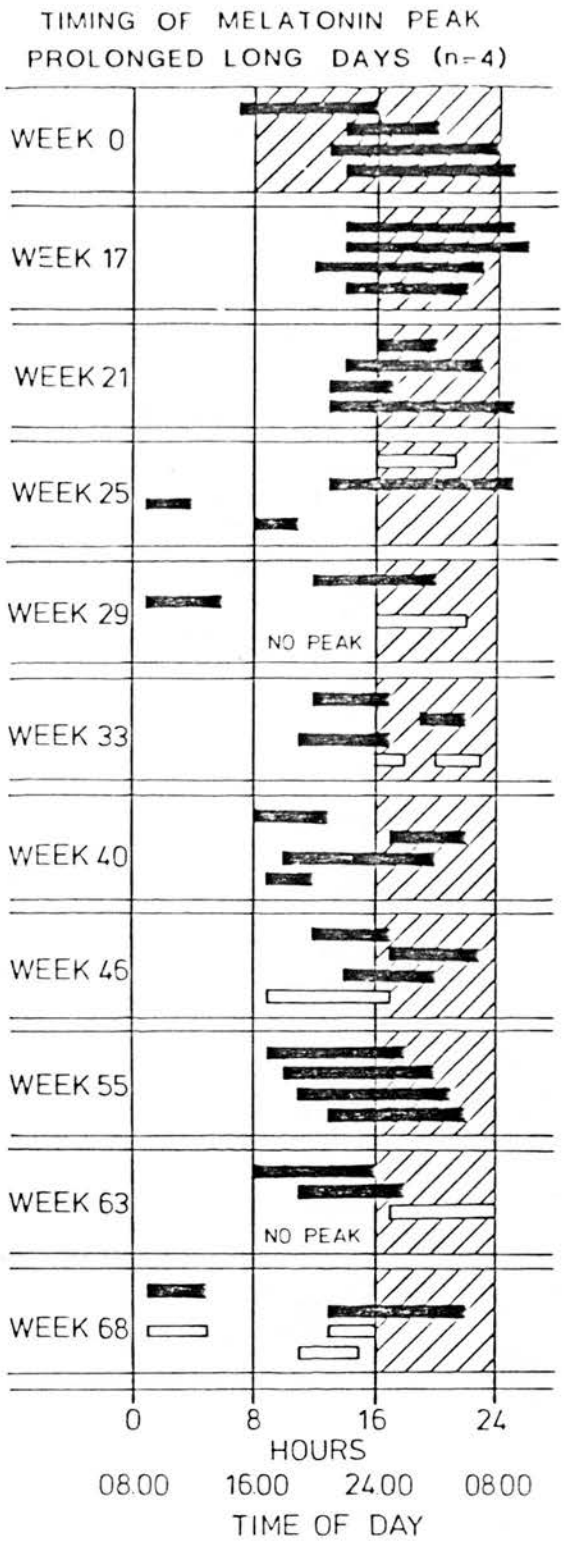


Fig. 4.4 Timing of the occurrence of peak MEL concentrations in th blood of 4 rams at various time intervals during exposure to a prolonged period of long daily photoperiods (16L:8D) after a 16 week period of short days (week 0; 8L:16D). Each bar in the same horizon plane represents a peak for a single animal (An open bar represents a period of high MEL levels which did not constitute a peak as defin in 2.6). The stippled areas represent periods of darkness.

characterised by peak hormone levels occurring at particular times of the day (Fig. 4.4). At the beginning of the study, these daily peaks consistently occurred late in the day, mainly during periods of darkness. However, this pattern was not maintained throughout the experiment, and in several instances, these peaks occurred early in the day, during periods of light; such asynchrony with the LD cycle was usually accompanied by a less consistent pattern between individual rams.

The large degree of variation observed in the blood MEL profiles of the 4 rams made it difficult to correlate these profiles with the long-term changes in testicular activity. However, during two periods of testicular regression (weeks 17-21 and 46-55 approximately), the MEL profiles were characterised by well-synchronised nocturnal peaks, while during two periods of spontaneous testicular development (weeks 25-40 and 63-68 approximately), the blood MEL profiles were disrupted.

(b) 'S group': The hourly fluctuations in the mean blood levels of MEL on 6 occasions during the exposure of rams to an extended period of short days are shown in Fig. 4.5. The 24h mean blood concentrations of MEL did not change significantly until after week 40, when they increased. These changes in the blood levels of MEL did not correlate with the long-term changes in the activity of the testes.

During their pre-treatment with long days (week 0), this group of rams showed consistent patterns of circulating MEL, with peak levels of this hormone being confined to the latter part of the day, during darkness (Fig. 4.6). The testes of this group were completely

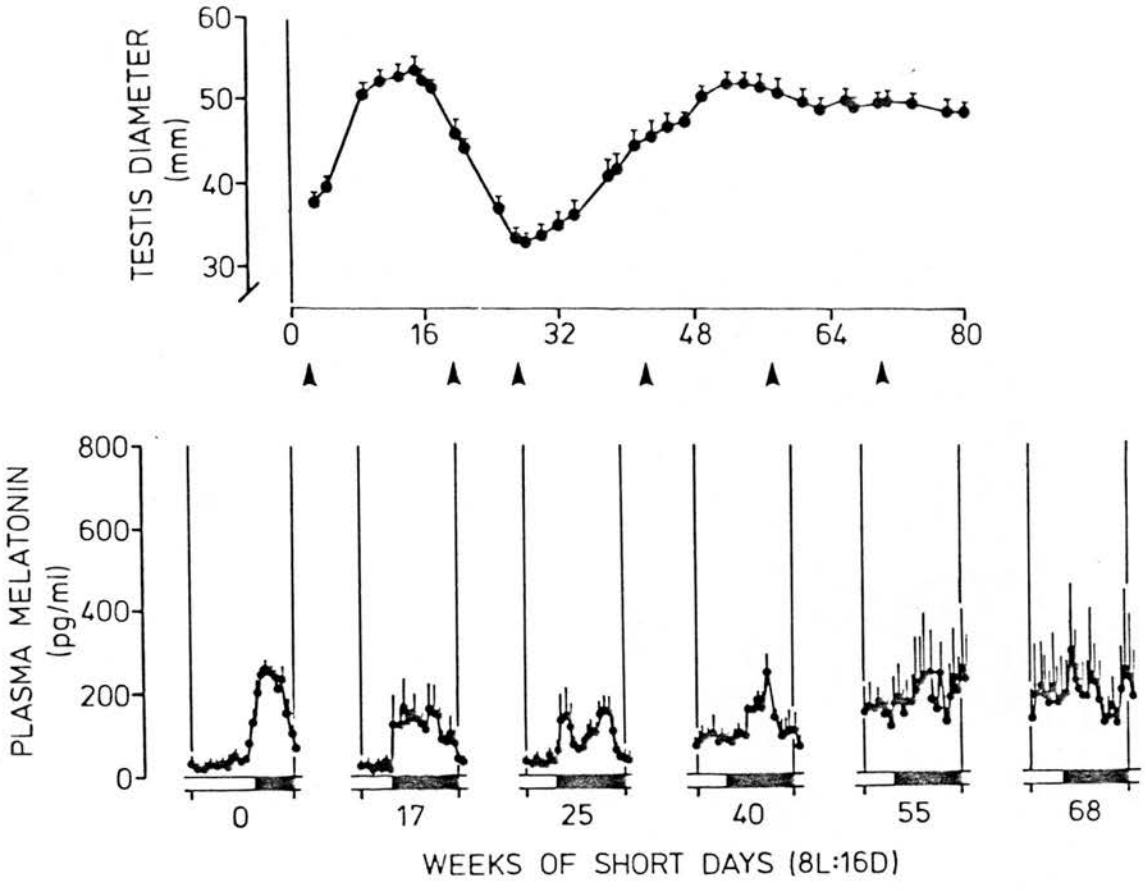


Fig. 4.5 Changes (mean ± S.E.M.) testis size and in the 24h pattern of melatonin concentrations in the blood plasma of 4 adult Soay rams exposed to a prolonged period of short daily photoperids (8L:16D). Before transfer to the 8L:16D regimes, the rams had been exposed to 16 weeks of long daily photoperiods (16L:8D). The data for week 0 is for the last day of that 16 week period of 16L:8D. The arrowheads refer to the points at which the hourly blood collections were made (weeks 0, 17, 25, 40, 55 and 68). The timing of periods of light (open bars) and darkness (closed bars) are shown at the bottom.

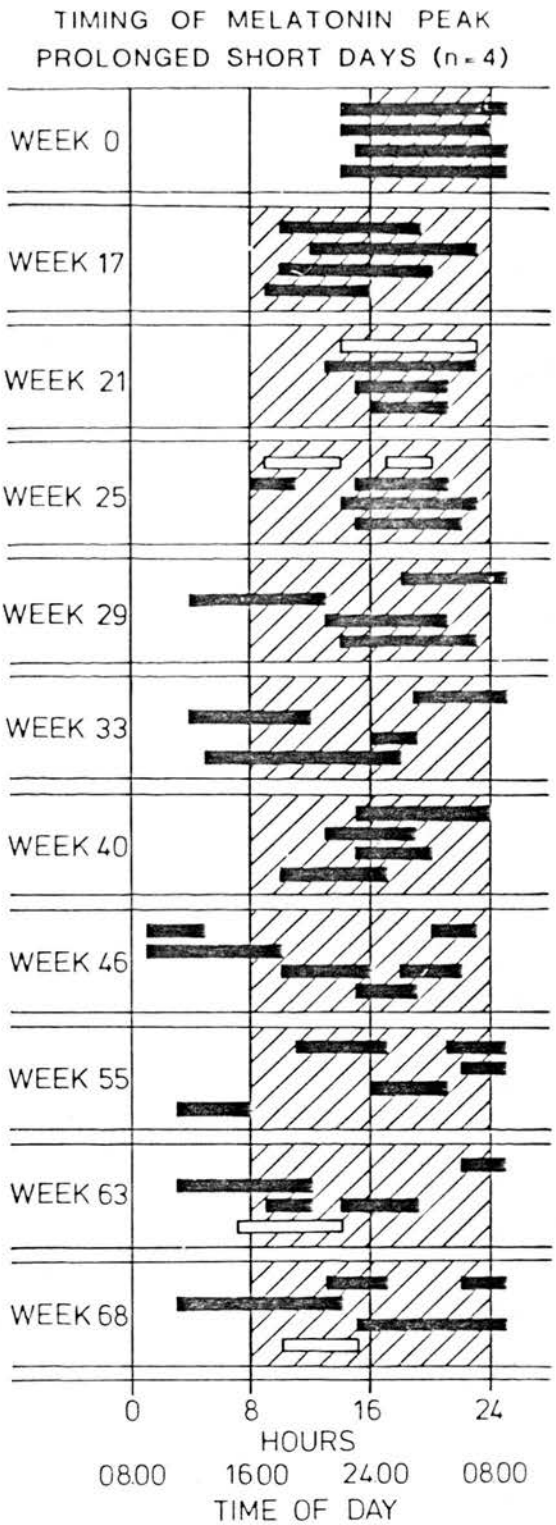


Fig. 4.6 Timing of the occurrence of peak MEL levels in the blood of 4 rams at various time intervals during exposure prolonged period of short daily photoperiods (8L:16D), after a 16 week period of long days (week 0, 16L:8D). Each bar in the same horizontal plane represents a peak for a single animal. (An open bar represents a period of high MEL levels which did not constitute a peak as defined in 2.6). The stippled areas represent periods of darkness.

regressed at that time. Subseqently, when the rams were transferred to short days, the MEL peaks occurred consistently earlier in the day, although still during darkness (week 17, Fig. 4.6); during this time testicular redevelopment had occurred. By week 21, when the testes had begun to regress, the MEL peaks occurred later in the day; this pattern, which resembled that seen during long days (week 0), persisted until week 29 when a disruption of the consistent blood patterns of MEL was observed. Spontaneous testicular redevelopment was initiated at around the same time when the disrupted MEL patterns were first noted (week 29); the testes were maintained at a near-maximal size for the remainder of the study, during which time the MEL profiles remained disrupted.

4.4 DISCUSSION

In the Soay ram, the testes regress during long days and grow during short days. Cycles of testicular activity can be induced in the laboratory by exposing rams to alternating 16 week periods of long days or short days (Lincoln & Short, 1980). In the present experiment, rams were held under either long days or short days for a period of nearly 2 years (94 weeks), and their reproductive activity was monitored. It was found that the rams held under both photoperiodic regimes could eventually over-ride the normal effects of the LD cycles to which they were exposed. Thus, those rams exposed to an extended period of long days were able to show cycles of testicular regression and redevelopment, despite the normally gonado-inhibitory photoperiod, while those rams exposed to short days for a prolonged period were, initially at least, able to regress their testes despite the normally gonado-stimulatory photoperiod. These observations therefore suggest that Soay rams become photorefractory.

The rates of testicular redevelopment during the exposure to

constant photoperiods in this study were retarded compared to those observed during alternating 16 week cycles of artificial photoperiods (c.f. Lincoln & Davidson, 1977; Chapter 3). Sluggish spontaneous redevelopment of the gonads has previously been found in other mammals believed to have become photorefractory (Reiter, 1972; Turek & Campbell, 1979; Johnston & Zucker, 1980a, Zucker, Johnston & Frost, 1980).

The fact that the long day-exposed group of rams continued to show gonadal cycles of activity, whereas the short day-exposed rams did not show such cycles (Figs. 4.1 & 4.2) is important, since it suggests that in addition to entraining the seasonal reproductive cycle, the photoperiod may play an active role in causing it. The reduced amplitude of the testicular cycles seen in the long day group (c.f. Lincoln & Davidson, 1977) may be taken as further evidence for this suggestion.

In two previous studies in which sheep were maintained under long or short photoperiods for prolonged periods, cycles in gonadal activity were observed (ewes: Ducker *et al*, 1973; rams: Howles *et al*, 1980). Those authors concluded that sheep may have an endogenous rhythm of gonadal activity which is normally entrained by the photoperiod so as to produce seasonal gonadal cycles. The recurring gonadal cycles obtained in the rams exposed to constant long daily photoperiods in this study would support the idea of an endogenous rhythm; however, the lack of cyclicity in those rams exposed to constant short daily photoperiods casts doubts on such a hypothesis. The lack of reproductive cyclicity during extended periods of exposure to short days is not unique to the present study; Cole (1953) found that Hampshire and Suffolk ewes showed continuous oestrous cycles when exposed to a prolonged period of short days.

In many species, photorefractoriness can only be broken by exposure to a photoperiod different to that which caused the loss in photosensitivity in the first place, e.g. most birds and small mammals which become refractory to the stimulatory effects of long days require exposure to non-stimulatory short days in order to restore their responsiveness to long days (Turek & Campbell, 1979; Hoffmann, 1981a). In contrast, species such as the ferret (Herbert, 1981) and sheep (Cole, 1953; Ducker et al, 1973 and Howles et al, 1980; Figs. 4.1 & 4.2), can apparently spontaneously emerge from the photorefractory condition, thus giving rise to the concept that endogenous cycles of reproductive activity exist. It has been proposed that there might be an asociation between the life-span of a species and its ability to recover from the photorefractory state (Herbert, 1981; Hoffmann, 1981a).

The PRL data obtained in the present study also support the idea that sheep become photorefractory. Cycles of PRL levels in the blood can normally be induced by subjecting rams to alternating 16 week cycles of long days and short days During long days, the blood PRL levels are elevated, while during short days, these levels are diminished (Lincoln, McNeilly & Cameron, 1978). In this present study, the blood PRL concentrations waxed and waned in those rams kept under constant long days, despite the continuance of the normally PRL-stimulatory photoperiod, while the rams kept under constant short days eventually showed increased blood PRL concentrations, despite the normally PRL-suppressive photoperiod. As with the changes in testicular activity, there was no common period in the cycles of blood PRL levels for the two groups of rams.

Although the 24h mean blood concentrations of MEL increased with

continued exposure to both constant long days and constant short days, there was no correlation between the absolute blood plasma levels of MEL and those of testicular activity. However, some correlations could be drawn between the blood patterns of MEL and the changes in testicular activity. In both groups of rams, consistent patterns in the blood levels of MEL were found until week 17 of the study, with peak levels of MEL always coinciding with periods of darkness (Figs. 4.4 and 4.6). When these peaks occurred during the last 8h of the day under long days, testicular regression was observed; however, when they occurred during the last 16h of the day under short days, testicular re-development was observed (c.f. Chapter 3). After week 17 of constant long days or constant short days, these blood patterns of MEL were frequently disrupted in both groups of rams (Figs. 4.4 & 4.6); these disrupted patterns were usually associated with periods during which spontaneous redevelopment of the testes was occurring. However, this correlation provides no clues as to whether the disrupted patterns are causal or symptomatic of the photorefractory state of the testes.

CHAPTER 5

TWENTY-FOUR HOUR RHYTHMS IN THE PLASMA LEVELS OF MELATONIN, PROLACTIN, SODIUM AND POTASSIUM AND THE REPRODUCTIVE RESPONSES OF RAMS EXPOSED TO TWO RESONANCE PHOTOPERIODS.

5.1. INTRODUCTION

Two main hypotheses exist to explain how organisms measure daylength. In one, it is proposed that time measurement depends on the absolute number of hours of light or dark in each 24 hour period ('hour-glass' model). In the other, time measurement depends on the way the daily photoperiod influences circadian rhythms generated endogenously by the animal (circadian hypothesis). The hour-glass model seems to hold for only a small number of insect species (Lees, 1966; Lees & Hardie 1981; Saunders, 1977), while support for the circadian model comes from experiments on a wide range of insects (Pittendrigh & Minis, 1964; Saunders, 1970), birds (Hamner, 1963; Follett & Sharp, 1969) and mammals (Elliott, Stetson & Menaker, 1972; Morin, Fitzgerald, Rusak & Zucker, 1977; Rusak & Zucker, 1979). These models are discussed at length by Elliott & Goldman (1981).

The involvement of a circadian mechanism was first proposed by Bünning in 1936 (Bünning, 1973). He suggested that organisms possess an endogenous circadian rhythm in their sensitivity to light, consisting of two half cycles (12 hours each), which he termed the "photophillic" and "scotophillic" phases. In long days (light period > 12hr), light impinges into the second half of the daily cycle (the light-sensitive phase) producing, in long-day species, a photo-induced response. An equivalent mechanism, albeit opposite, can be envisaged to operate for short-day species. Pittendrigh & Minis (1964) extended Bünning's hypothesis into an "external coincidence model" in which light has a dual role: (a) that of inducer (or suppressor), and (b) that of an entraining agent for endogenous circadian rhythms. Those authors also proposed an "internal coincidence model" in which light only serves to entrain the endogenous rhythms, induction being the result of internal phase-relationships of specific rhythms.

Nanda & Hamner (1958), studying flower formation in the short-day soybean (Glycine max), devised what is still considered to be the best test for the involvement of circadian rhythms in the photoperiodic response. Their protocol consisted of measuring the photoperiodic response under a range of light/dark (LD) cycles in which a fixed period of light was coupled to dark periods of varying duration (e.g. 6L:6D, 6L:12D, 6L:18D, 6L:24D....6L:66D). The rationale behind this experimental design is that if the photoperiodic clock is based on the hour-glass principle, none of the LD cycles listed would be expected to induce a long day response. On the other hand, if photoperiodic induction was found to rise and fall as a periodic function of the LD cycle (modulo 24h), it would be inferred that neither the duration of light, nor the duration of darkness, nor the L/D ratio is the determining factor. Such a result would argue strongly in favour of a circadian mechanism (Elliott, 1981; Elliott & Goldman, 1981). Nanda & Hamner (1958) observed that maximal responses were obtained when the LD cycle lengths equalled 24h, 48h and 72h, and that cycle lengths of 18h, 36h and 60h produced minimal responses.

Resonance schedules have been used widely in the study of photoperiodism in birds (Hamner, 1963, 1964; Follett & Sharp, 1969; Follett, Mattocks & Farner, 1974), but in only two mammalian species (golden hamsters: Elliott, Stetson & Menaker, 1972; Stetson, Elliott & Menaker, 1975; field voles; Grocock & Clarke, 1974). In this chapter, a simple resonance photoperiod experiment carried out on Soay sheep is described. Because of the large size of the animals it was only practicable to use two groups of 8 rams. After exposing both groups to long daily photoperiods (16h light: 8h darkness, 16L:8D) to induce testicular regression, one group was exposed to a 48h cycle comprising

8L:40D, and the other group to a 36 h cycle comprising 8L:28D. It was predicted that these schedules would give contrasting reproductive responses, i.e. 8L:40D simulating a 'short day' and 8L:28D simulating a 'long day'. For an insight into the mechanisms involved in the photoperiodic response of sheep, daily fluctuations in the blood levels of MEL, PRL and electrolytes (sodium and potassium) were measured during exposure to the two resonance photoperiods. The changes in MEL concentration would reflect changes in pineal activity, those in PRL, changes in hypothalamic dopaminergic activity, and those in electrolytes, changes in feeding, metabolic and adrenocorticoid activity.

5.2 MATERIALS AND METHODS

Animals and lighting: Sixteen, sexually mature rams of the Soay breed (aged 1-2 years) which had been living out-of-doors near Edinburgh (56°N) since birth, were brought into light-proof sheds in November, 1978. They were held under artificial illumination of long days (16L:8D) for a period of 16 weeks so as to induce complete testicular regression (Lincoln & Short, 1980). Eight of the rams were then exposed to a resonance photoperiod of 8L:40D for 16 weeks (referred to as the 8L:40D group). The other 8 rams were exposed to a lighting regimen of 8L:28D for 16 weeks (referred to as the 8L:28D group). The animals were fed a commercial pelletted diet (AA6, Animal Breeding Research Organisation, Edinburgh) given once daily at 09.00 hours throughout the study. The temperature in the animal sheds was partially controlled using heaters at night to reduce daily fluctuations.

Reproductive indexes and hormone and electrolyte assays: The diameter of the testes of the rams were measured at weekly intervals, and on

each occasion the inguinal sexual skin flush was recorded on an arbitrary scale of 0-5 (Lincoln & Davidson, 1977).

On 4 occasions during the study, blood samples were collected at hourly intervals for periods of 2-4 days (Day 0-4, 28-29, 78-79, and 111-112 after the switch from long days to the resonance photoperiods). For this, an indwelling cannula was inserted into the jugular vein of each ram during one of the 8 h periods of light, at least 24 h before the beginning of the collection of blood samples. The blood plasmas were stored at -20°C until required for the assay of melatonin and prolactin by the methods detailed in Sections 2.5.1 and 2.5.2 respectively. The concentrations of sodium and potassium were also measured in some of the samples (4 rams from each treatment group) by flame spectrophotometry. The precision of the assay for the sodium concentrations was $\pm$ 1 mmol/l and for the potassium concentrations, $\pm$ 0.1 mmol/l. The electrolyte determinations were made by the Department of Clinical Chemistry, Royal Infirmary of Edinburgh.

This study was performed in collaboration with Dr. G.A. Lincoln, and preliminary data from it have already been published (Lincoln, Almeida & Arendt, 1981; Lincoln & Almeida, 1982).

5.3 RESULTS

Testicular size and sexual flush: The changes in the reproductive parameters in the rams during exposure to the two resonance photoperiods are summarised in Fig. 5.1. At the beginning of the study, all of the rams were reproductively quiescent after the 16 weeks of long days (16L:8D). The testes were regressed and none of the animals had an inguinal sexual flush.

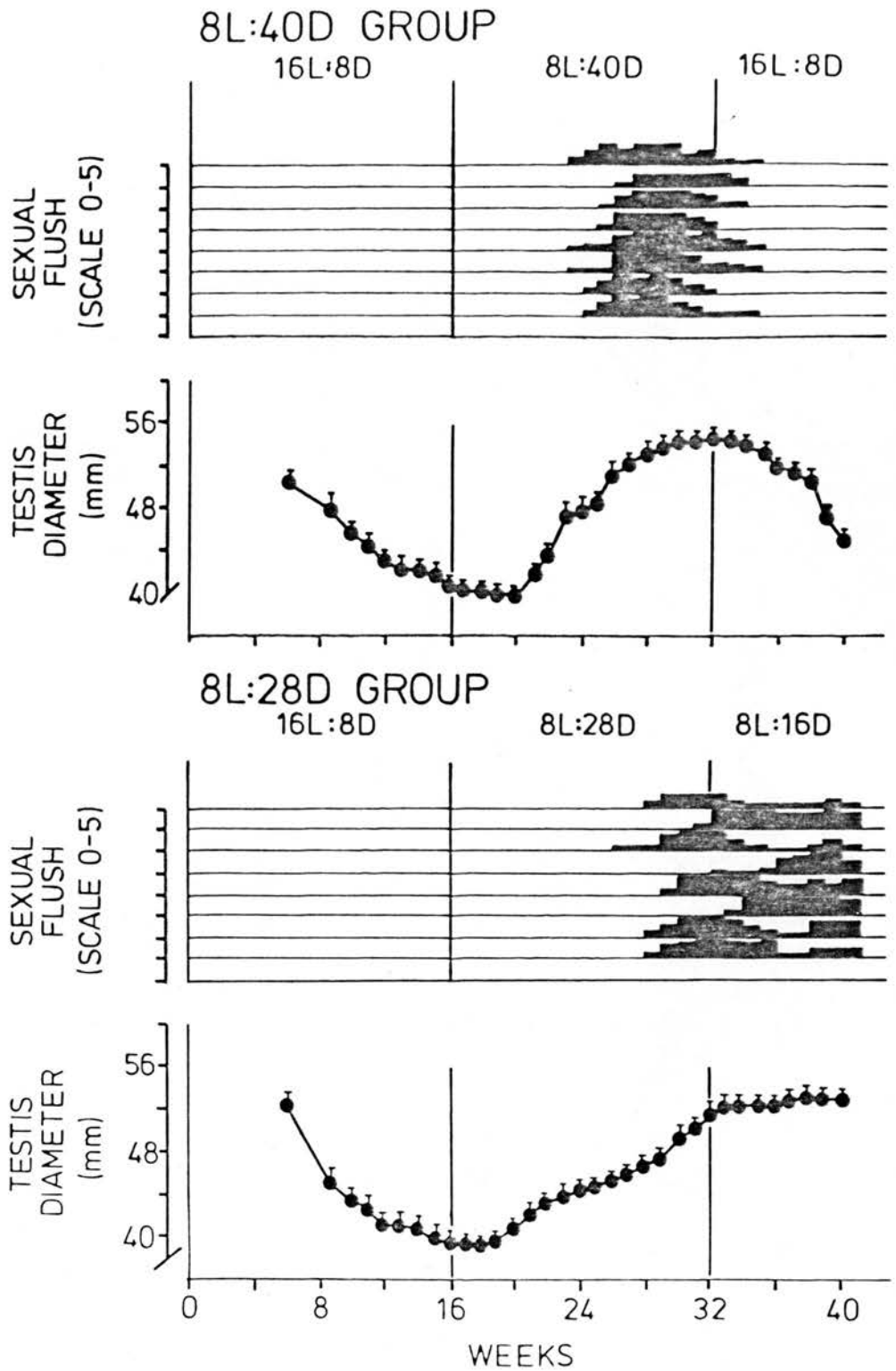


Fig. 5.1 Changes in the diameter of the testes (mean $\pm$ SEM) and the intensity of the sexual skin flush (individual data) in groups of 8 adult Soay rams exposed to a resonance light cycle of either 8L:40D (upper panel) or 8L:28D (lower panel) for 16 weeks following exposure to long days (16L:8D) for 16 weeks. Note the more rapid sexual development under the 8L:40D regimen.

During exposure to the resonance photoperiods both groups of rams showed a resurgence of reproductive activity. The testes enlarged and there was a reappearance of the sexual skin flush. The timing of these reproductive changes differed, however, between the two groups. The animals exposed to the 8L:40D lighting regimen developed their testes more rapidly than the animals under the 8L:28D regimen (analysis of variance: $p < 0.001$, $F=81.7$, $df=1,112$), and showed significantly earlier development of the sexual skin flush. The 8L:40D group also differed from the 8L:28D group in showing greater synchrony in the occurrence of each of the reproductive changes. For example, the sexual flush first appeared during a 3 week period (weeks 7-10) after the onset of the 8L:40D regimen, while this change occurred during a 9 week period (weeks 10-19) after the onset of the 8L:28D regimen.

Hormone concentrations: The changes in the mean plasma concentrations for MEL and PRL during the two resonance photoperiods are shown in Table 5.1. The mean daily concentrations of MEL were increased, while the mean daily concentrations of PRL were markedly decreased, during exposure to both the 8L:40D and 8L:28D regimens. There were differences between the two groups, however, in the timing of the changes in the PRL levels. A significant decrease in PRL levels occurred by day 28-29 in the 8L:40D group compared to day 111-112 in the 8L:28D group. There was no significant difference between the groups in the long term changes in the mean blood plasma levels of MEL.

Melatonin, prolactin and electrolyte rhythms: The hourly fluctuations in the plasma levels of MEL and PRL on various occasions during exposure to the 8L:40D and 8L:28D regimens are shown in Figs. 5.2 and 5.3. The hourly changes in the plasma concentrations of potassium and sodium paralleled each other and so only the potassium data are shown

TABLE 5.1

Summary of the changes in the concentration of melatonin and prolactin in the blood plasma from two groups of 8 rams sampled on 5 occasions following transfer from long days (16L:8D) to a resonance lighting regimen of either 8L:40D or 8L:28D. The results are the mean ± S.E.M. of 24h median hormone values based on blood samples collected at hourly intervals for each day

	DAY 0 (16L:8D)	DAYS 1-3	DAYS 28-29	DAYS 78-79	DAYS -
MELATONIN (pg/ml)					
8L:40D Group	25.3 ± 8.3	37.5 ± 9.1	71.8 ± 10.8[a]	-	79.8 ± 16.5[a]
8L:28D Group	38.3 ± 13.9	39.3 ± 7.4	109.1 ± 24.5[a]	101.2 ± 22.2[a]	127.0 ± 25.1[a]
PROLACTIN (ng/ml)					
8L:40D Group	67.6 ± 11.6	69.7 ± 14.1	20.8 ± 10.4[a,b]	1.5 ± 0.8[a,b]	1.5 ± 0.8[a]
8L:28D Group	71.9 ± 10.6	77.0 ± 14.2	77.0 ± 14.2	67.6 ± 12.3	3.7 ± 1.8[a]

a = Significantly different from corresponding values for Day 0; Student 't'-test, $P \leq 0.05$

b = Significantly different from corresponding values for 8L:28D Group
Student 't'-test, $P \leq 0.05$

here (Fig. 5.4).

On day 0 (last day of long days of 16L:8D), the plasma profiles for MEL, PRL and potassium were similar in both groups, with peak levels for all three compounds occuring late in the light phase or during darkness.

During each period of blood sampling under the 8L:40D photoperiod there was evidence of consistent 24 h rhythms in the plasma levels of MEL and the electrolytes. The highest values were observed during darkness, following the 8 h light period, and again during darkness, approximately 24 h later (Figs. 5.2 and 5.5). While peak blood MEL values occurred relatively earlier during the subjective day under the 8L:40D regimen compared to under the 8L:16D regimen (Fig. 5.5), peak blood levels of the electrolytes occurred at the same time of day under both these photoperiodic regimens (Fig. 5.4). At the beginning of the exposure to the 8L:40D treatment, there was evidence of a consistent 24 h rhythm in the plasma levels of PRL (Fig. 5.3). By day 28-29, however, the levels of this hormone had become too low to detect any rhythmical changes in its secretion.

In the group of rams treated with the 8L:28D photoperiod, the blood profiles of MEL became irregular by day 28-29, and remained so for the rest of the study (Fig. 5.2); peaks of MEL were found during both periods of light and darkness, and the period between peaks was seldom close to 24 h (Fig. 5.6). The blood profiles of PRL also became irregular from day 28-29 onwards (Fig. 5.3). The plasma profiles of the electrolytes remained unchanged from the 16L::8D pattern throughout the experiment (Fig. 54.). Comparisons between the periods in the rhythms of blood MEL, PRL and the electrolytes in the 8L:40D and 8L:28D groups are shown in Table 5.2.

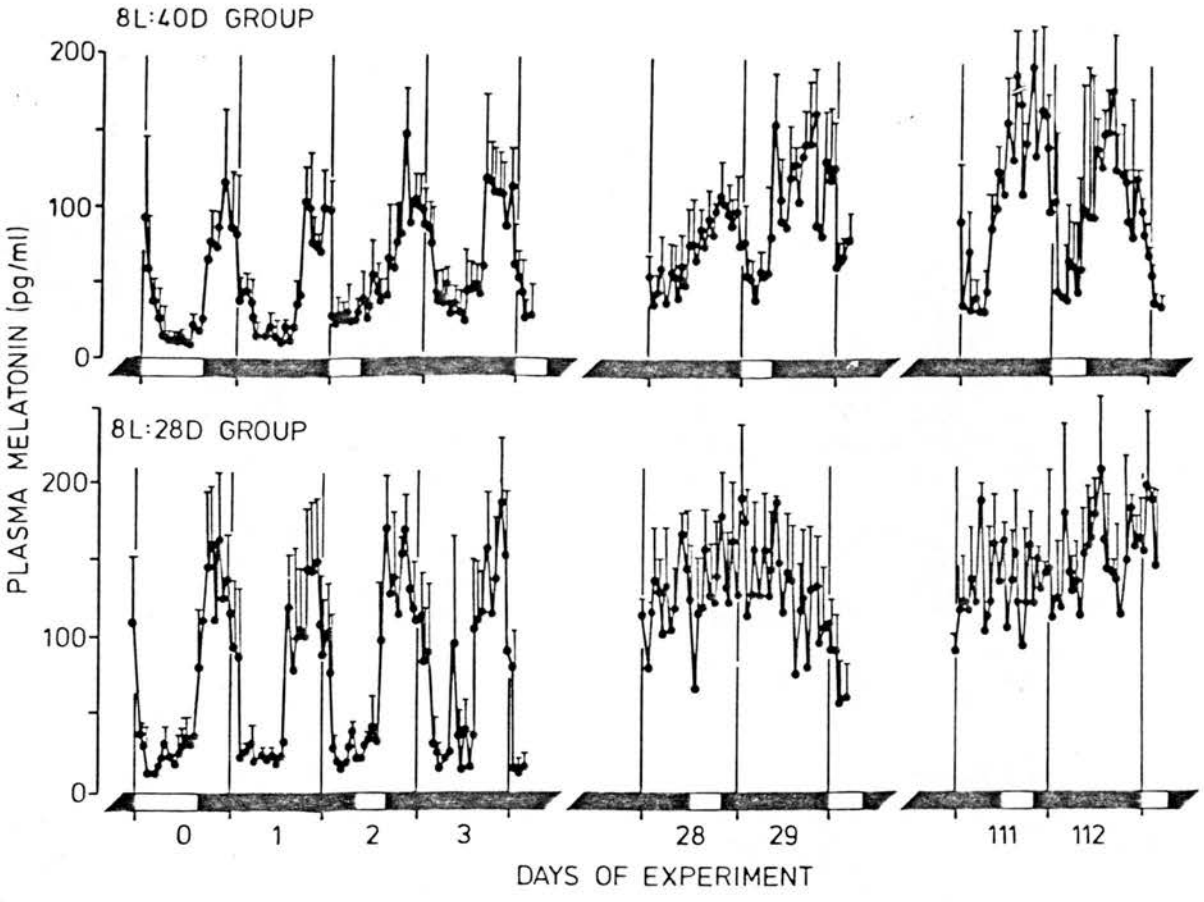


Fig. 5.2 Changes in the concentration of melatonin (mean ± SEM) in the blood plasma from 8 adult Soay rams sampled at hourly intervals for 2-4 days on 3 occasions during exposure to 16 weeks of either 8L:40D (upper panel) or 8L:28D (lower panel), following 16 weeks of long days (16L:8D). Day 0 is the last day of long days. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

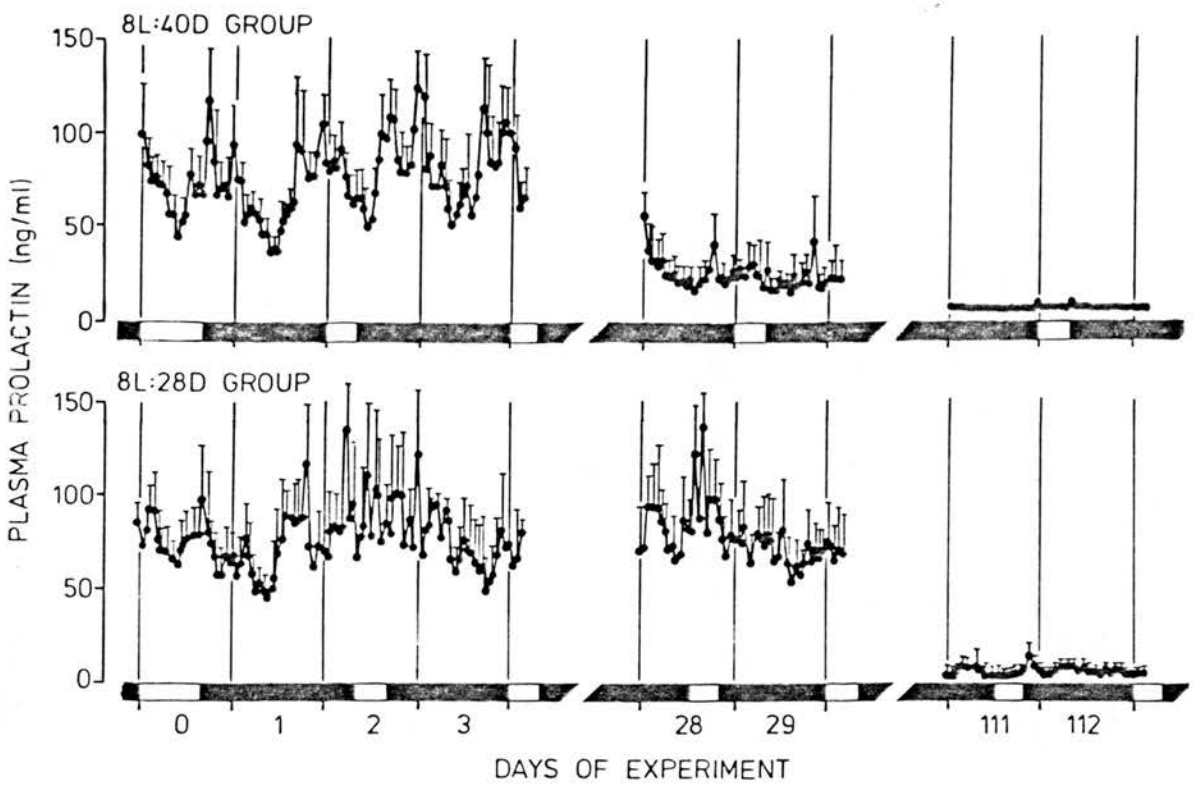


Fig. 5.3 Changes in the concentration of prolactin (mean ± SEM) in the blood plasma from 8 adult Soay rams sampled at hourly intervals for 2-4 days on 3 occasions during exposure to 16 weeks of either 8L:40D (upper panel) or 8L:28D (lower panel), following 16 weeks of long days (16L:8D). Day 0 is the last day of long days. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

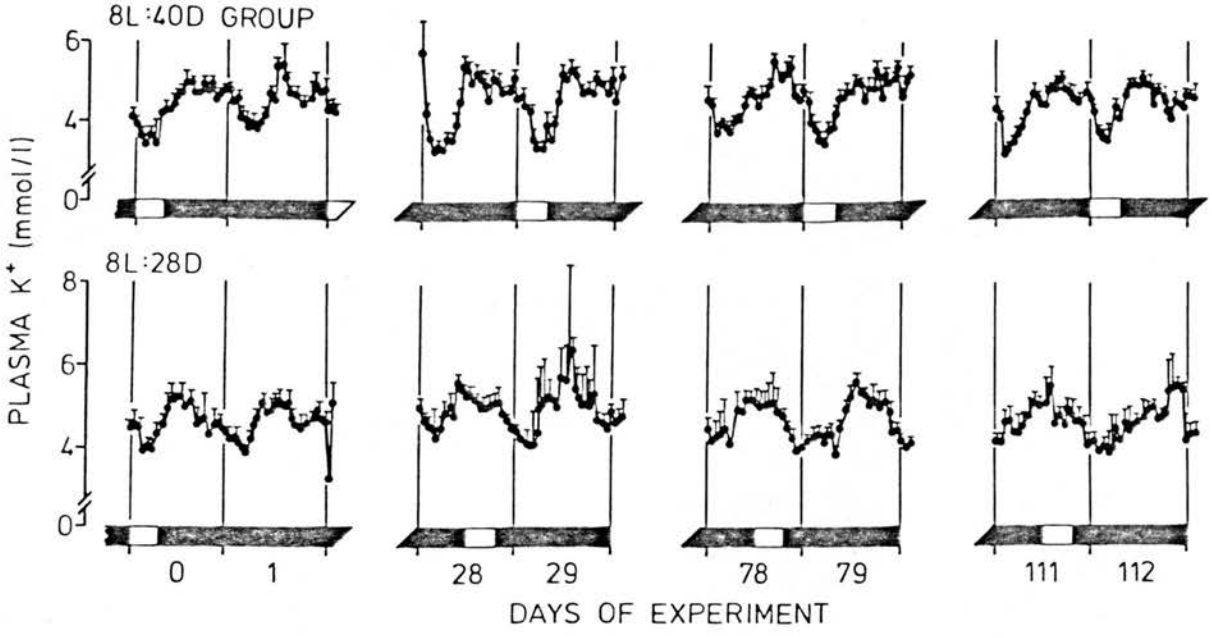


Fig. 5.4 Changes in the concentration of potassium (mean ± SEM) in the blood plasma from 4 adult Soay rams sampled at hourly intervals for 2 days on 4 occasions during exposure to 16 weeks of 8L:40D (upper panel) or 8L:28D (lower panel), following 16 weeks of 16L:8D. Day 0 is the last day of 16L:8D. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

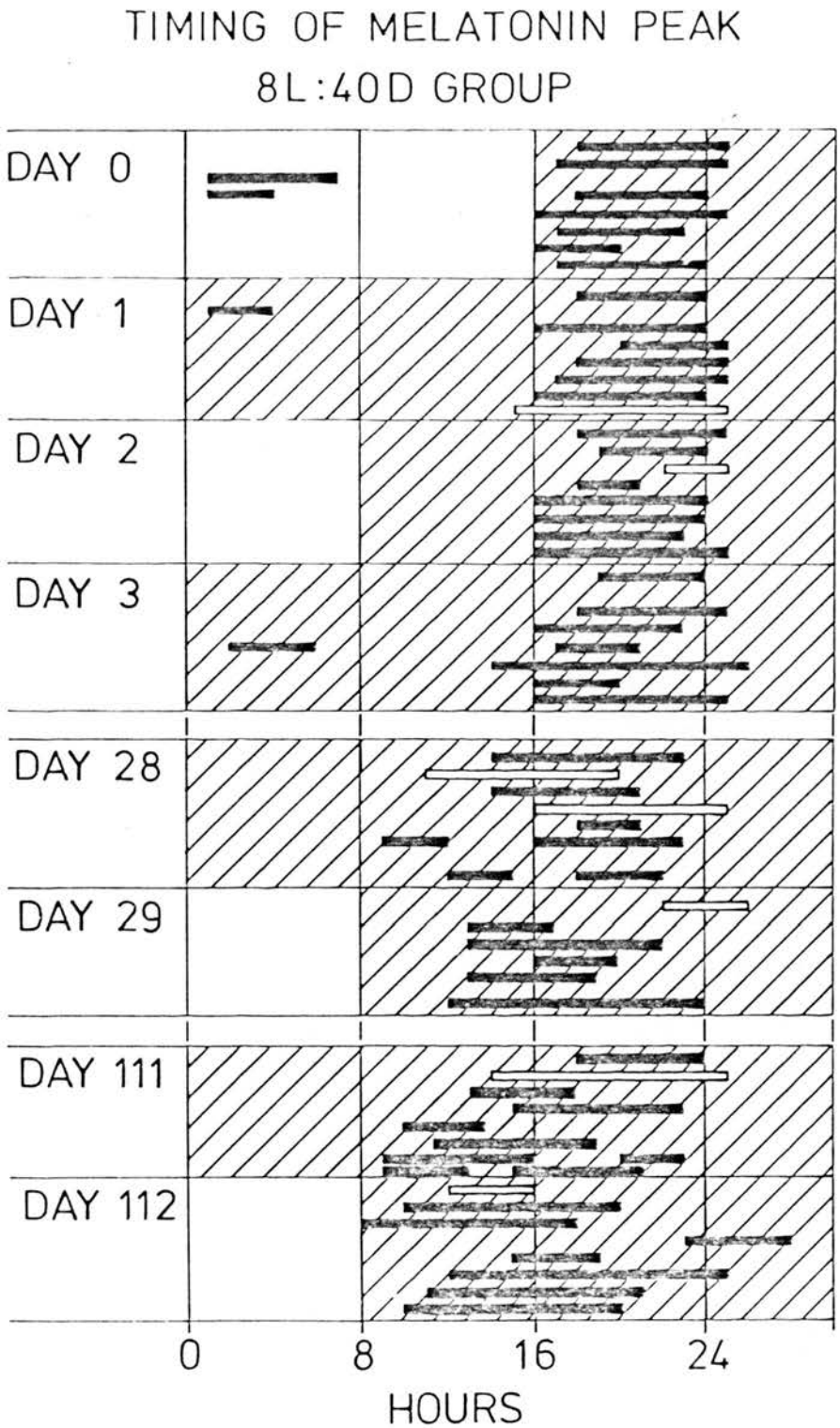


Fig. 5.5 The timing of peaks in the plasma concentrations of melatonin in 8 adult Soay rams based on blood samples collected at hourly intervals for 2-4 days on 3 separate occasions during exposure to 16 weeks of 8L:40D, following a period of 16 weeks 16L:8D. Day 0 is the last day of 16L:8D. Each solid horizontal bar represents the duration of a significant peak for an individual animal while an open bar represents a peak which does not include a significant outlier and is shown for completeness. Periods of darkness are indicated by diagonal shading.

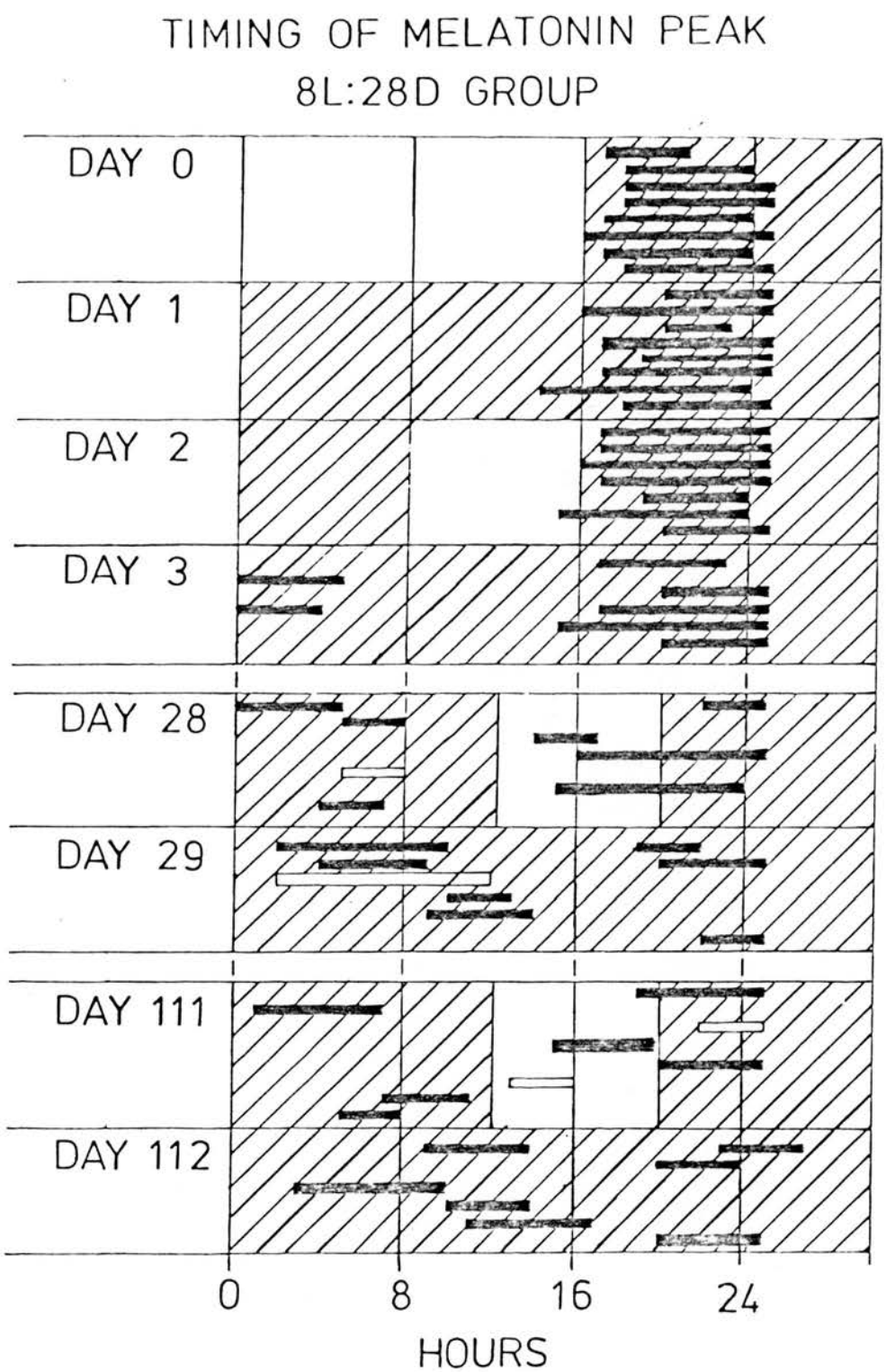


Fig. 5.6 Timing of peaks in the plasma concentrations of melatonin in 8 adult Soay rams based on blood samples collected at hourly intervals for 2-4 days on 3 separate occasions during exposure to 16 weeks of 8L:28D, following a period of 16 weeks of 16L:8D. Day 0 is the last day of 16L:8D. Each solid horizontal bar represents the duration of a significant peak for an individual animal while an open bar represents a peak which does not include a significant outlier and is shown for completeness. Periods of darkness are indicated by diagonal shading.

TABLE 5.2

Periods of cycles in the concentrations of melatonin, prolactin, sodium and potassium in the blood collected from Soay rams on three occasions during exposure to either 8L:40D or 8L:28D photoperiods. The period lengths quoted are the median $\pm$ range for all the values obtained for each treatment group.

COMPOUND	TREATMENT (n)	CYCLE LENGTH (h)		
		DAYS 0-3	DAYS 28-29	DAYS 111-112
Melatonin	8L:40D (8)	24 (20-30)	23 (21-32)	25 (18-32)
	8L:28D (8)	25 (18-30)	21 (12-52)	39 (12-52)
Prolactin	8L:40D (8)	24 (19-31)	24 (18-25)	Undetectable
	8L:28D (8)	25 (12-31)	31 (17-52)	29 (10-52)
Sodium	8L:40D (4)	25 (20-26)	25 (23-27)	25 (25-26)
	8L:28D (4)	24 (19-26)	24 (24-26)	23 (22-23)
Potassium	8L:40D (4)	22 (18-22)	25 (23-27)	24 (22-24)
	8L:28D (4)	23 (22-27)	26 (20-27)	26 (23-27)

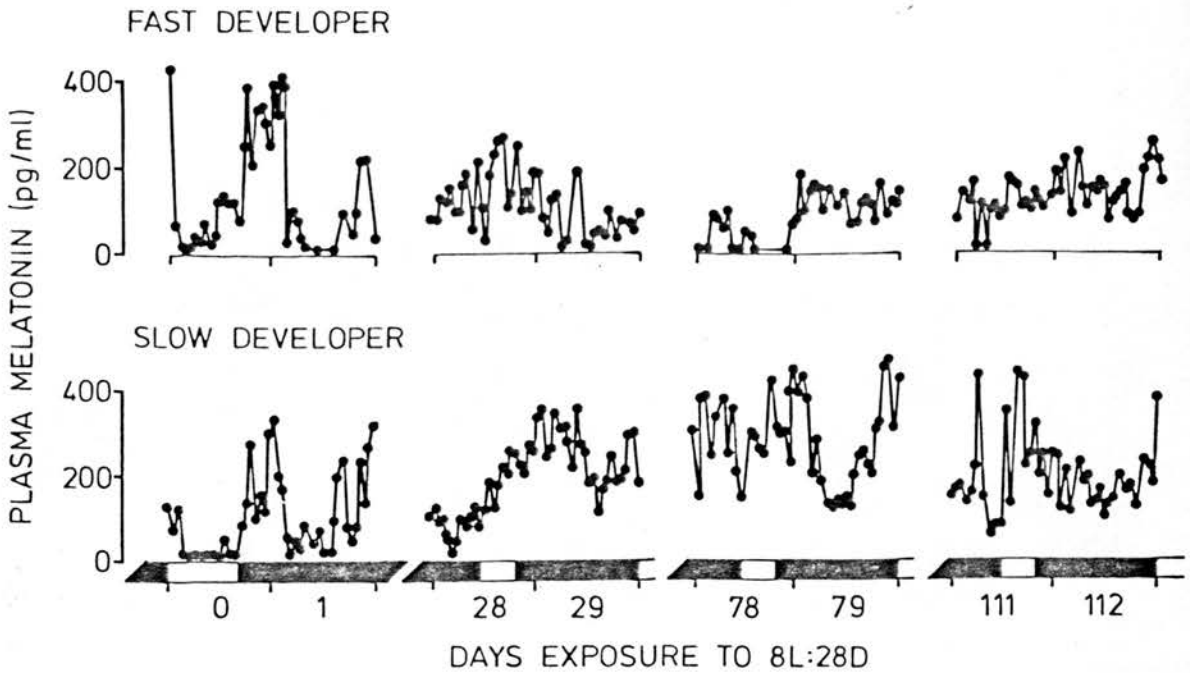


Fig. 5.7 Changes in the concentration of melatonin in the plasma of two adult Soay rams based on blood samples collected at hourly intervals for 2 days on 4 occasions during exposure to 16 weeks of 8L:28D, following a period of 16 weeks of 16L:8D. Day 0 is the last day of 16L:8D. The animals were selected to represent a fast developer (upper panel) and a slow developer (lower panel) based on the rate of growth of the testes and the timing of the reappearance of the sexual skin flush. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

Individual variation in the 8L:28D group

Under the 8L:28D regimen there was considerable variation between the individual rams in the timing of their testicular changes and rhythmic patterns of hormone secretion. This allowed some correlations to be tested. There was a significant correlation between the rate of growth of the testes and the timing of the development of the sexual flush (Spearman Rank Correlation: $r=0.90$, $p < 0.01$). There was a significant correlation between the timing of the increase in the size of the testes and the decrease in the 24 h mean plasma concentration of prolactin (Spearman Rank Correlation: $r=-0.70$, $p < 0.05$), but no significant correlation with the changes in the 24h mean concentrations of melatonin. There were, however, no apparent correlations between the hour to hour variations in the plasma levels of melatonin and prolactin and the rates of testicular growth and rhythms in hormone secretion. An example of the hormone data for the ram which showed the most rapid development of the testes and earliest sexual flush from the 8L:28D group is shown in Figure 5.7 for comparison with similar data for the slowest ram in that group.

5.4 DISCUSSION

All the rams used in this study had been exposed to long days of 16L:8D for 16 weeks prior to the start of the experiment so as to induce reproductive quiescence. Those rams transferred from 16L:8D to the 8L:40D resonance schedule showed a rapid and synchronised reactivation of their reproductive organs, as gauged by testicular diameter and the onset of the sexual skin flush (Fig. 5.1). The rate and magnitude of this response was similar to that observed when rams were previously transferred from long days of 16L:8D to short days of 8L:16D (Chapter 3). In contrast, the rate of reproductive

redevelopment in the rams exposed to the 8L:28D regimen was sluggish and redevelopment commenced at different times in the individual rams (Fig. 5.1).

The testicular recrudescence of the 8L:28D group might at first appear to be a stimulated response to the 8L:28D photoperiod, since all the animals in that group eventually showed a redevelopment of their testes. However, it will be recalled from the previous chapter that rams exposed to non-stimulatory long days (16L:8D) for a period greater than 16 weeks, spontaneously re-grow their gonads; the rate of testicular redevelopment found under the 8L:28D schedule is, in fact, similar to that found in rams during prolonged exposure to long days. This suggests that the apparent response to the 8L:28D regimen was in effect spontaneous redevelopment, similar to photorefractoriness. This is in contrast to the changes seen under the 8L:40D regimen, where a stimulated response definitely occurred. Since the change from long days to either 8L:16D, 8L:28D and 8L:40D all involve a reduction in the absolute daily number of hours of light, and only the 8L:16D and 8L:40D regimens gave a stimulated response, these results argue against a simple hour-glass model; they suggest that a circadian mechanism underlies the photoperiodic control of reproduction in rams.

Differences were also found between the two groups of rams in their PRL responses to the two resonance photoperiods (Fig. 5.3). The decline in blood PRL levels was much more rapid under the 8L:40D regimen than under the 8L:28D regimen. The rate of decline in PRL under the 8L:40D photoperiod was similar to that previously observed when rams were transferred from long days of 16L:8D to short days of 8L:16D (Chapter 3). These PRL data are inversely correlated with the testicular changes, and also argue against a simple hour glass model

being involved in the photoperiodic response of the sheep.

The short-term fluctuations in the blood levels of MEL, PRL and the electrolytes may provide some insight into the mechanisms involved in the measurement of photoperiodic time in sheep. In particular, the daily changes in the blood levels of MEL were different during exposure to the 8L:40D and 8L:28D regimens. At the beginning of the experiment, there was a consistent 24h rhythm in the blood levels of MEL in both groups of rams, with MEL peaks occurring during the last 8h of the subjective day, during darkness. Under the 8L:40D regimen, this 24h rhythm persisted. Peaks of MEL in the blood were well synchronised between the individual rams in the group and occurred principally during darkness on all the occasions studied. In contrast, under the 8L:28D regimen, there was little evidence of a 24h rhythm. Peaks of MEL in the blood were poorly synchronised between the individual animals in the group, and were not confined to periods of darkness; these disrupted blood patterns of MEL persisted until the end of the study.

One explanation for these results is that the 24h rhythm in MEL secretion is generated endogenously by some circadian clock, and that this rhythm represents the basis of the photoperiodic response. Since the 8L:40D treatment represents a total cycle length of 48h, which is a multiple of 24h, the light period in each cycle comes at the same time relative to the endogenous circadian rhythm; entrainment of the endogenous rhythm is thus possible (Elliott & Goldman, 1981), and MEL peaks occur in the blood at 24h intervals. On the other hand, the 8L:28D regimen represents a total cycle length of 36h, and the light period in each cycle comes at different times relative to the circadian rhythm; entrainment of the endogenous rhythm is thus not possible, and peak blood levels of MEL are found at irregular intervals.

The hourly fluctuations in the blood levels of PRL were more difficult to analyse because the overall levels were already markedly reduced by day 28-29 in the rams under the 8L:40D regimen, and the rams under the 8L:28D regimen which had detectable levels, showed much individual variation Nevertheless, the blood PRL patterns differed between the two groups (Table 5.2).

Consistent hourly fluctuations in the blood levels of the electrolytes measured were observed throughout the study in both groups of rams. At the beginning of the study, a clearly defined 24h rhythm was found in the blood patters of sodium and potassium, with peak levels occuring relatively late in the subjective day. This pattern was maintained under both the 8L:40D and 8L:28D schedule, This result was taken to indicate that the prevailing photoperiod does not have a direct effect on the blood electrolyte rhythm and that the time of feeding influences this rhythm in sheep. (In this study, all the rams were fed at 0900h every day; at this time, the blood levels of sodium and potassium were always at their nadir).

Taken together, the present data suggest that there is an underlying circadian mechanism to the photoperiodic response of rams, and that the control of the pattern of MEL secretion may be part of this mechanism.

CHAPTER 6

PATTERNS OF MELATONIN AND PROLACTIN SECRETION IN SOAY RAMS EXPOSED TO 10 DAYS OF EITHER CONSTANT LIGHT OR CONSTANT DARKNESS

6.1 INTRODUCTION

In the previous chapters it has been shown that under ordinary 24h LD cycles, the Soay ram displays a 24h rhythm in its blood plasma concentrations of MEL. Evidence which suggested that this rhythm might be a circadian rhythm was also obtained in an experiment using resonance photoperiods (Chapter 5). Circadian rhythms are normally entrained by the environmental LD cycle to have a period of precisely 24h. However, by definition, circadian rhythms are endogenously generated rhythms and, in the absence of entraining agents, they continue to be expressed, i.e. they "free-run" with a period close to 24h (Elliott & Goldman, 1981).

As a preliminary test of whether the Soay sheep could free-run its 24h rhythm in blood MEL concentrations, adult rams were exposed to 10d of constant light (LL) or constant darkness (DD), and their hourly blood levels of MEL were measured. The changes in the blood levels of PRL were also measured as these levels were previously found to provide a useful index of how a given photoperiod is interpreted by sheep (see Chapter 3). Since the anatomical pathway by which photic information is conveyed from the eyes to the pineal is known to include the superior cervical ganglion (SCG) in a number of mammals (Lincoln, 1979a,b; Rusak & Zucker, 1979; Reiter, 1980; Cardinali, 1981), rams which had their SCG surgically removed were also included in the study.

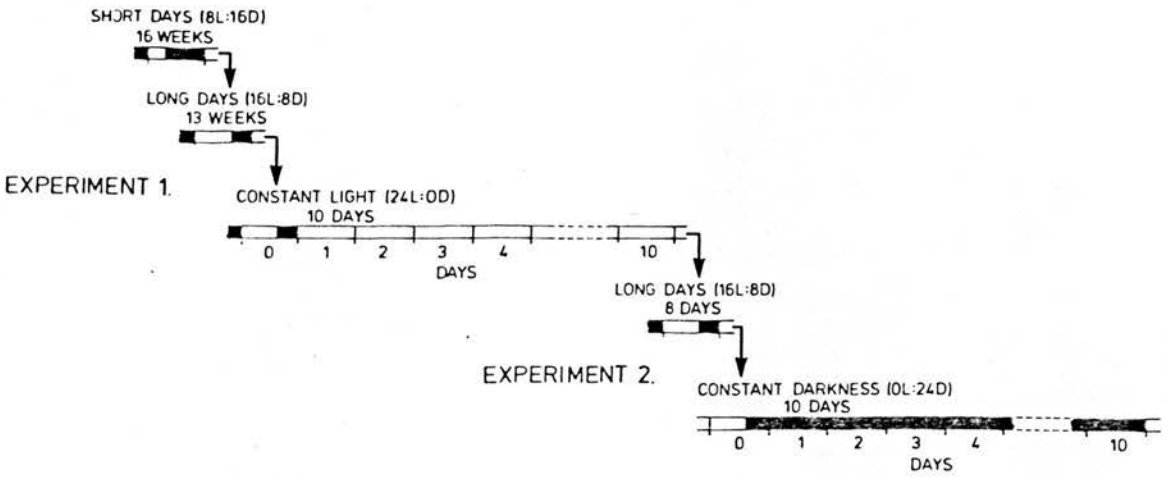


Fig. 6.1 Experimental design used for studying the effects of exposure to conditions of constant light or constant darkness upon the blood plasma concentrations of melatonin and prolactin in intact and SCG_X Soay rams.

6.2 MATERIALS AND METHODS

Animals: Eight adult Soay rams which had been exposed to alternating 16 week periods of long days (16L:8D) and short days (8L:16D) during the previous 3 years were used in this study. The superior cervical ganglia (SCG) of 4 of the rams had been surgically removed 3 years prior to this study (see 2.4), and one of the control rams received the sham operation at that time. All 8 rams were housed together in a light-proof shed in which the daily period of illumination always commenced at 0800 hours. Food and water were always provided between 0800 and 0900 hours and this pattern of maintenance (see 2.1) was continued during the course of the present experiments.

Experiment 1: The experiment was started during the 13th week of a period of long daily photoperiods (16L:8D). After 93 days of long days, the rams were exposed to 10d of continuous light (LL). The intensity of light was the same as that used during the previous period of long days (approximately 160 lux at the level of the sheeps' heads). Hourly blood samples were obtained from the rams during the last day of the long daily photoperiod (day 0) and days 1,2,3 and 10 of the LL treatment (Fig. 6.1). These samples were collected through cannulae inserted into the jugular veins of the rams at least 16 hours prior to the commencement of the sample collections (see 2.3). The blood plasmas were stored at -20°C until assayed for their concentrations of MEL and PRL by the RIA methods described in 2.4 and 2.5 respectively.

Experiment 2: Following the 10d treatment with LL, the rams were once again subjected to 16L:8D for a period of 9 days. They were then exposed to 10d of constant darkness (DD), (Fig. 6.1). During DD, cannulation and blood collections were performed with the aid of a 15W

TABLE 6.1

Mean (± SEM) blood plasma concentrations of MEL in adult Soay rams transferred from long days (16L:8D, Day 0) to 10d of constant light (LL, Experiment 1) or constant darkness (DD, Experiment 2).

EXPERIMENT	GROUP (n)	24h MEAN BLOOD MEL CONCENTRATION (pg/ml)			
		DAY 0	DAY 1	DAY 2	DAY 10
(1) LL	Intact (4)	56.6 ± 23.2	48.8 ± 29.6	68.9 ± 25.4	44.8 ± 18.5
	SCGx (4)	28.6 ± 8.5	21.3 ± 9.1	29.5 ± 11.7	27.8 ± 13.5
(2) DD	Intact (4)	67.6 ± 25.9	78.4 ± 26.5	88.8 ± 23.4	107.5 ± 30.3
	SCGx (3)	31.6 ± 15.0	19.6 ± 6.8	32.7 ± 14.9	61.1 ± 16.2

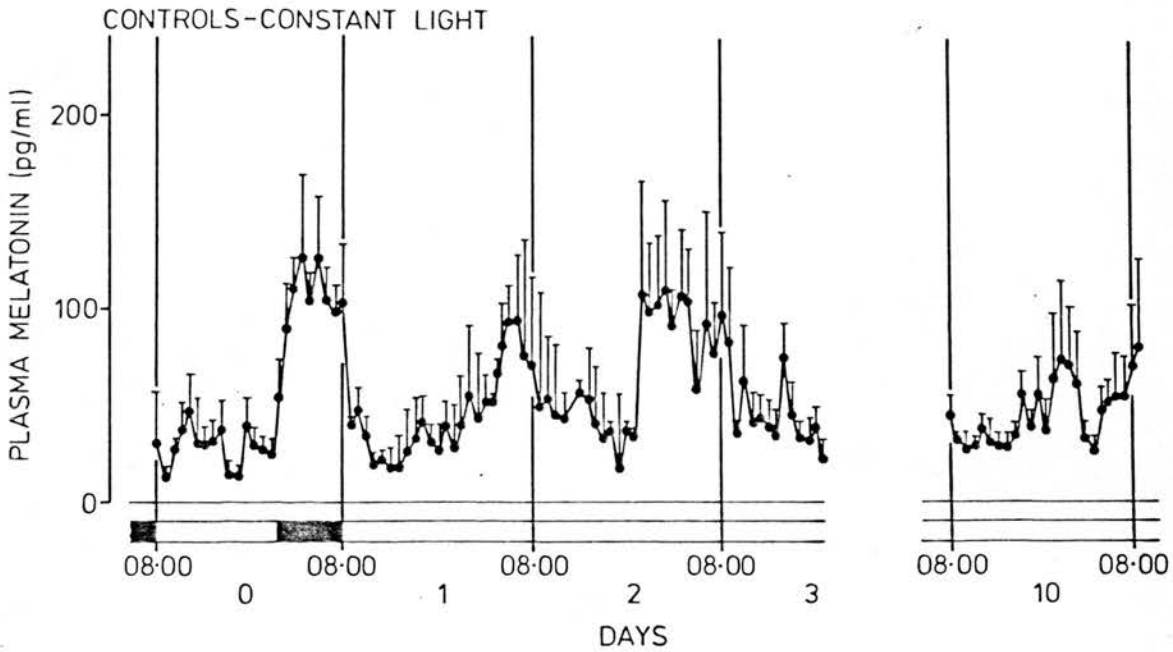


Fig. 6.2 Hourly changes in the concentration of melatonin (mean ± S.E.M.) in the blood plasma of 4 adult Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L:8D) to one of constant light (24L:0D or LL). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

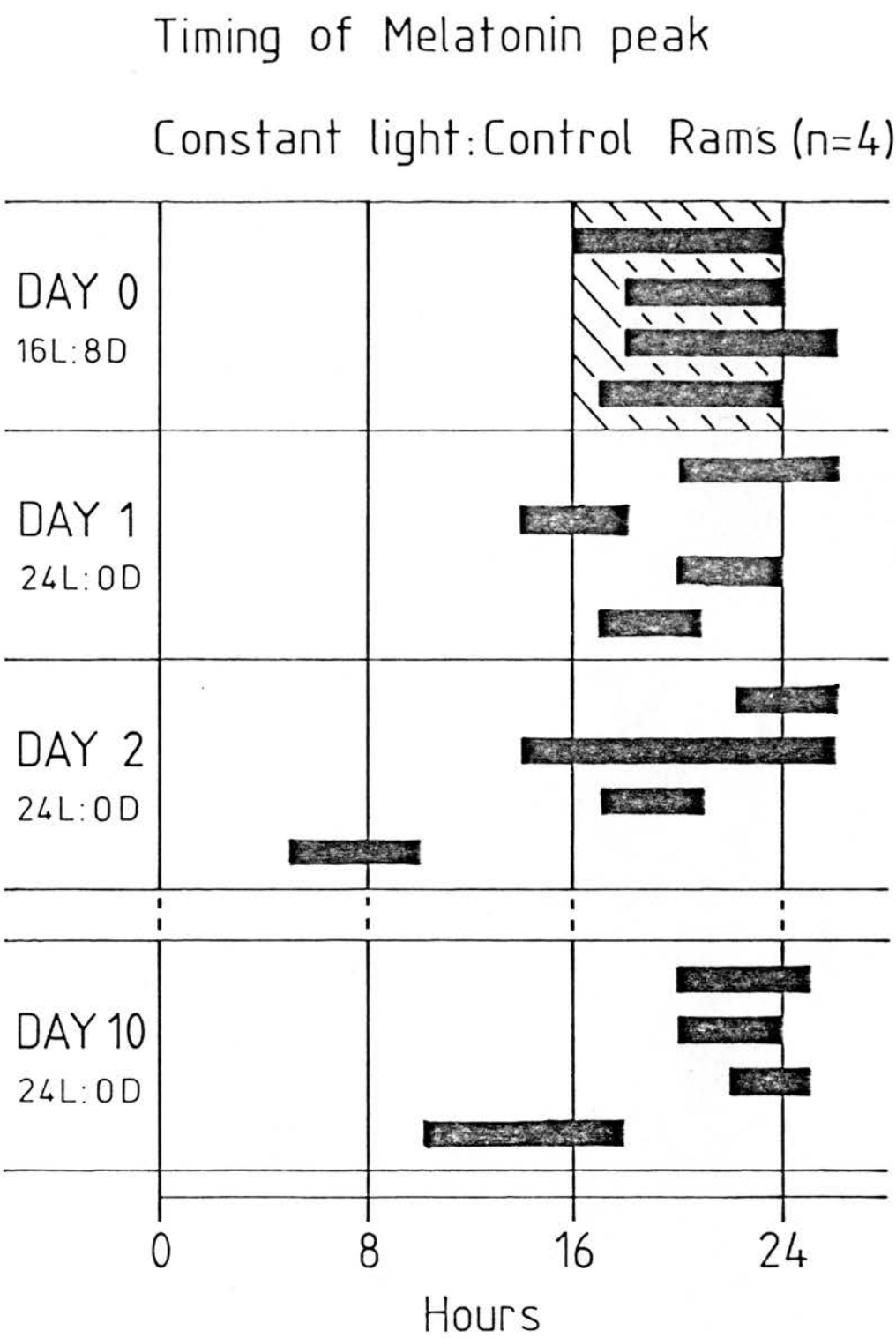


Fig. 6.3 Timing of peaks in the blood plasma concentrations of melatonin in 4 adult Soay rams transferred from a period of long days (16L:8D) to one of constant light (24L:0D or LL). Day 0 is the last of the long days period. Each solid horizontal bar represents the duration of a significant peak for an individual ram. Periods of darkness are indicated by diagonal shading.

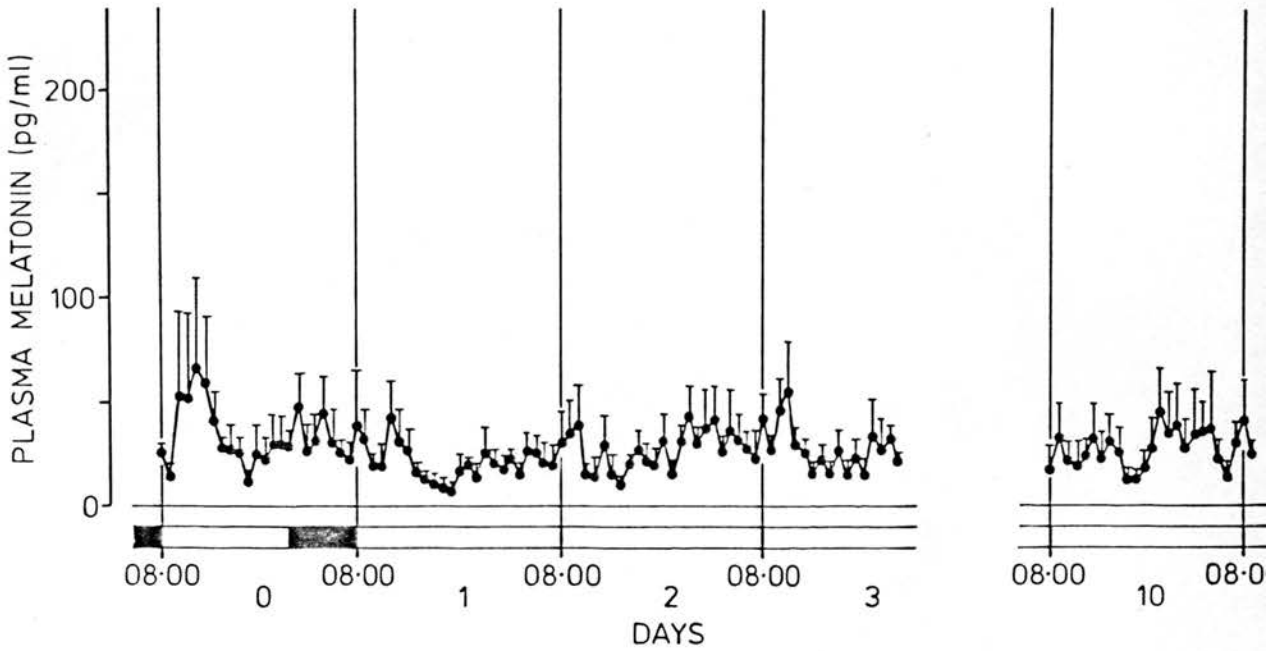


Fig. 6.4 Hourly changes in the concentration of melatonin (mean ± SEM) in the blood plasma of 4 adult SCGx Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L:8D) to one of constant light (24L:0D or LL). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

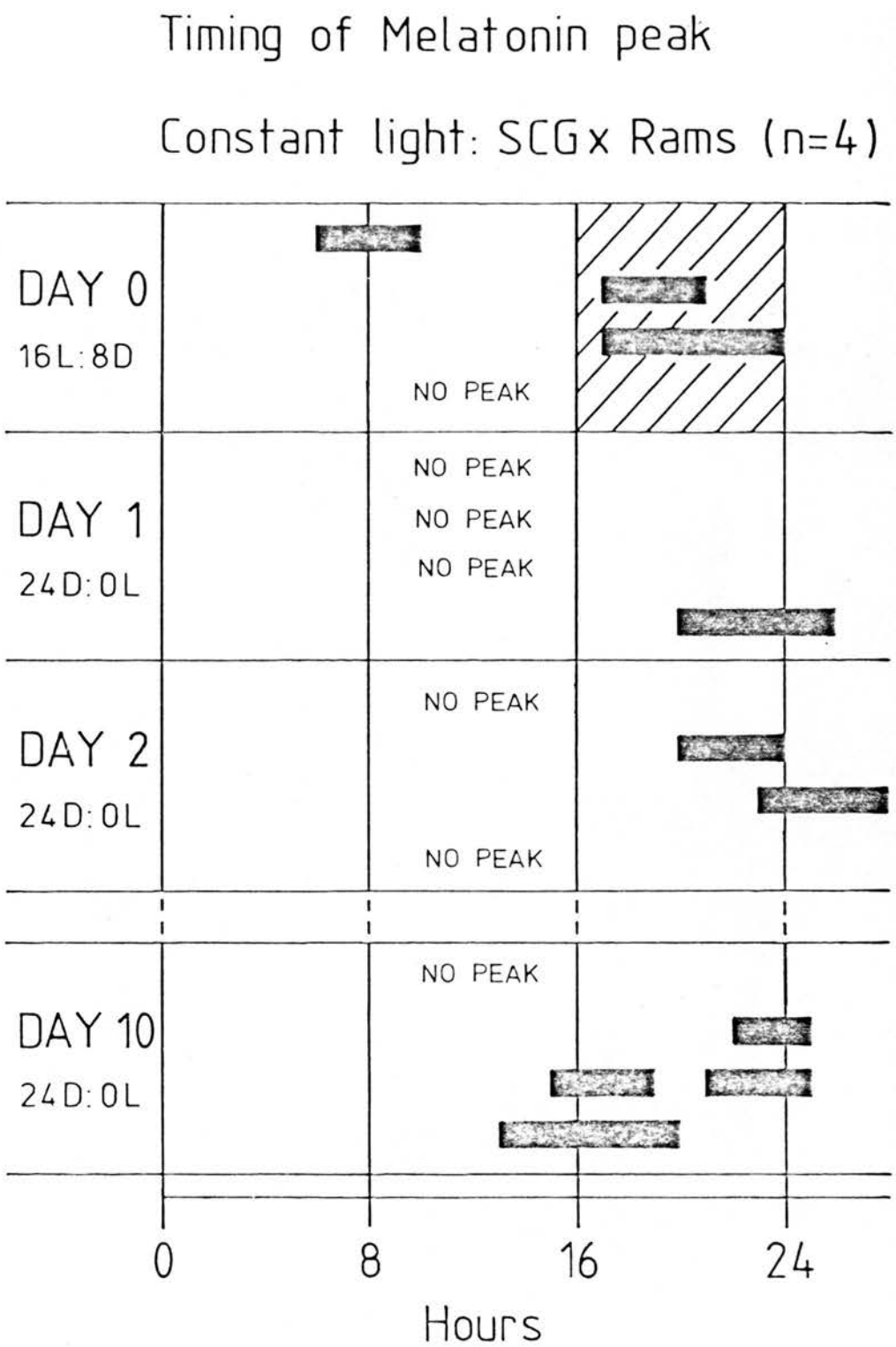


Fig. 6.5 Timing of peaks in the blood plasma concentrations of melatonin in 4 adult SCGx Soay rams transferred from a period of long days (16L:8D) to one of constant light (24L:0D or LL). Day 0 is the last day of the long days period. Each solid horizontal bar represents the duration of a signi peak for an individual ram. Periods of darkness are indicat by diagonal shading.

red light bulb (Philip's) and a hand-held torch which emitted a point source of light (<10 lux). The latter was used sparingly, and care was taken not to direct it at the eyes of the sheep. Hourly blood samples were collected and assayed for MEL and PRL on the last long day (Day 0) and on days 1,2,3 and 10 of the DD treatment. On day 10, only 3 of the SCGx rams were successfully blood sampled.

6.3 RESULTS

6.3.1 Experiment 1: Exposure to LL for 10d

Blood MEL concentrations: The exposure of intact rams to 10d of LL did not result in any significant changes in the 24h mean blood levels of MEL (Table 6.1). There were, however, significant peaks in the MEL levels on each occasion when the animals were sampled (Fig. 6.2). On the last long day (16L:6D, day 0), all 4 intact rams showed peak blood concentrations of MEL during the last 8h of the day during darkness (Fig. 6.3). This pattern was more-or-less maintained during the 10d of LL, with 3 of the rams showing peak levels of MEL in the blood; the fourth control animal showed peak blood levels of MEL earlier in the day.

The exposure of SCGx rams to 10d of LL did not result in any significant changes in the 24h mean blood levels of MEL. Throughout the study these levels remained significantly lower than those in the intact rams (Table 6.1). Peak blood levels of MEL were also found in this group, but their occurrence was irregular and inconsistent between individual rams (Figs. 6.4 & 6.5).

Blood PRL concentrations: In the control rams exposed to LL, there was a significant decline in the blood PRL levels by Day 10 (Table 6.2). In the SCGx rams exposed to this photoperiod, there was no significant change in the blood levels of PRL (Table 6.2). The blood levels of PRL

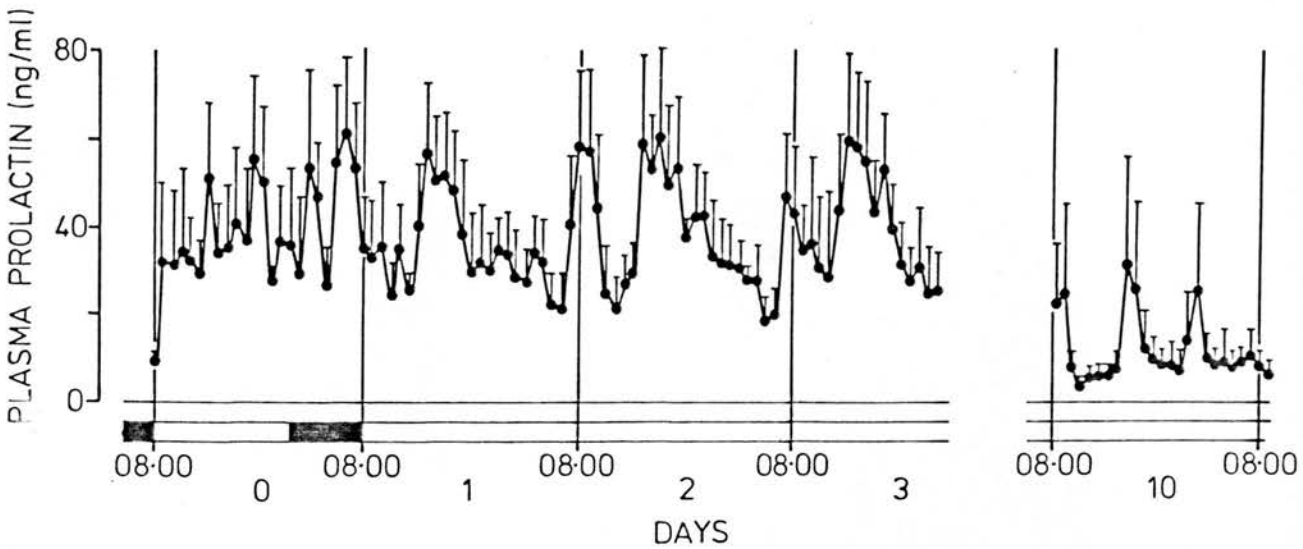


Fig. 6.6 Hourly changes in the concentrations of prolactin (mean ± SEM) in the blood plasma of 4 adult Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L:8D) to one of constant light (24L:0D or LL). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

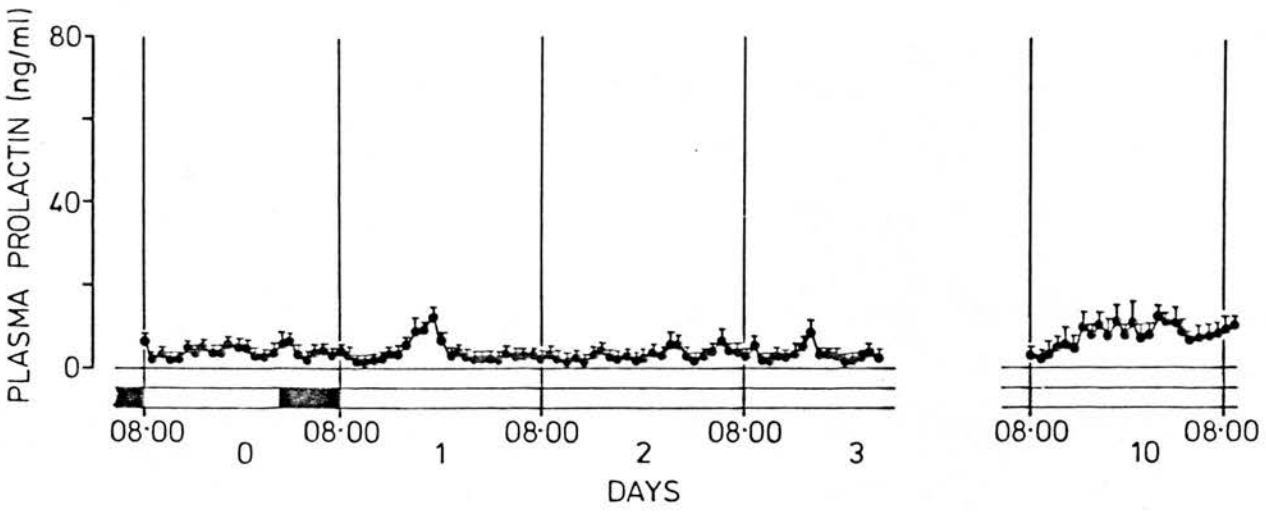


Fig. 6.7 Hourly changes in the concentration of prolactin (mean ± SEM) in the blood plasma of 4 adult SCGx Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L:8D) to one of constant light (24L:0D or LL). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

TABLE 6.2

Mean (± SEM) daily blood plasma concentrations of PRL in adult Soay rams transferred from long days (16L:8D, Day 0) to 10d of constant light (LL, Experiment 1) or constant darkness (DD, Experiment 2).

EXPERIMENT	GROUP (n)	24h MEAN BLOOD PRL CONCENTRATION (ng/ml)			
		DAY 0	DAY 1	DAY 2	DAY 10
(1) LL	Intact (4)	53.6 ± 74.6	36.9 ± 12.7	37.1 ± 12.6	11.7[a] ± 9.7
	SCG x (4)	3.9 ± 1.2	3.7 ± 1.7	4.8 ± 5.4	8.0 ± 2.9
(2) DD	Intact (4)	16.1 ± 7.4	12.3 ± 6.6	10.2 ± 5.4	6.3[a] ± 4.1
	SCG x (3)	4.7 ± 4.8	3.4 ± 3.6	2.2 ± 1.1	5.6 ± 2.9

a $p < 0.05$; significantly different compared with Day 0 for same group in each experiment.

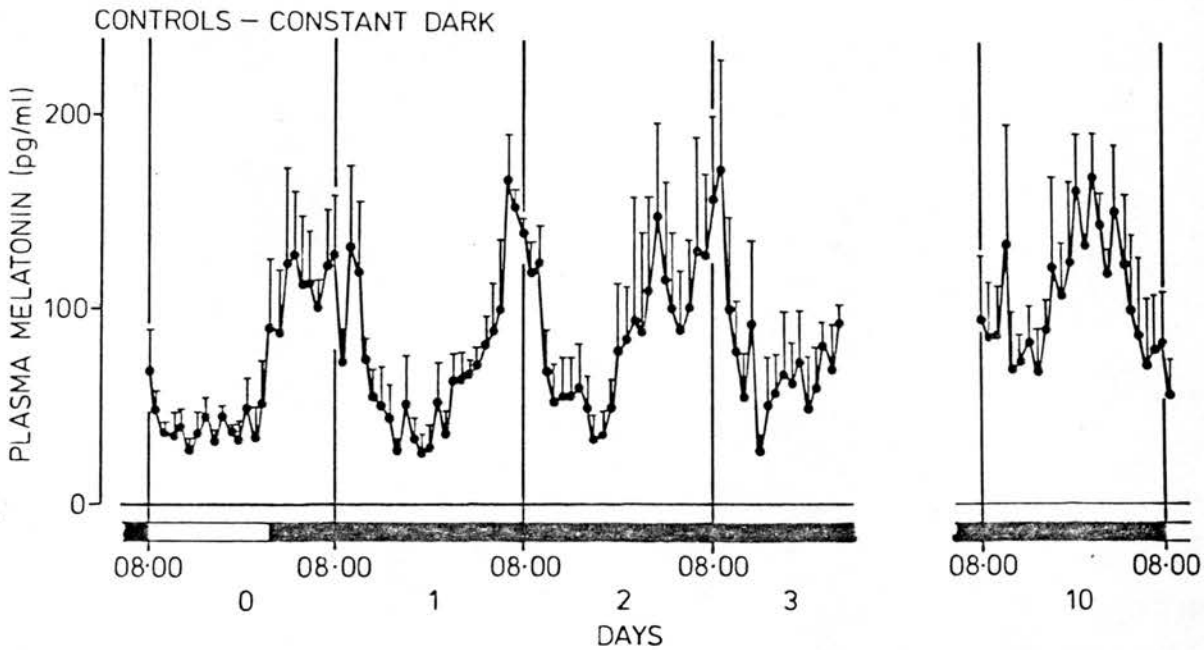


Fig. 6.8 Hourly changes in the concentration of melatonin (mean ± S.E.M.) in the blood plasma of 4 adult Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L:8D) to one of constant darkness (0L:24D or DD). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

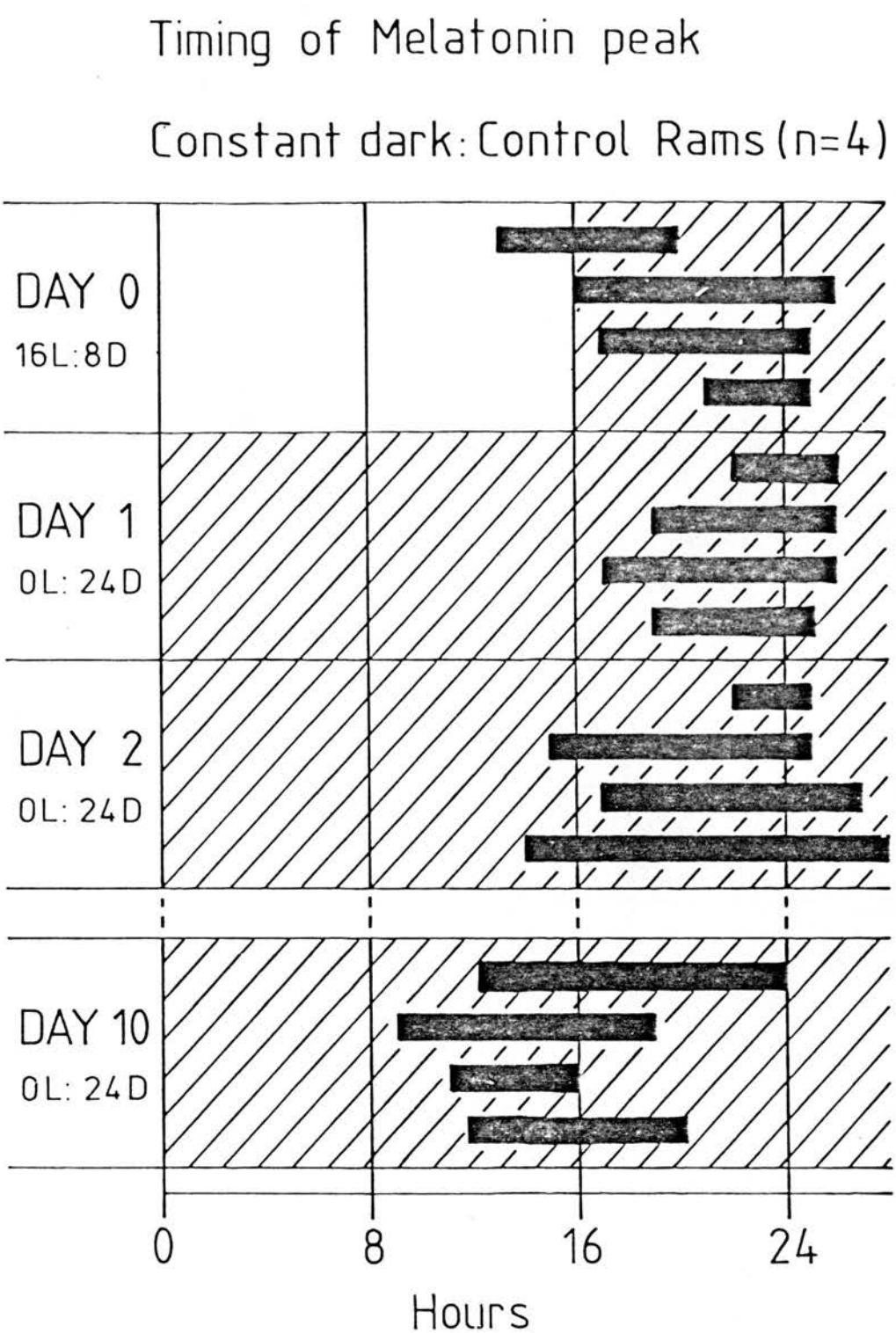


Fig. 6.9 Timing of peaks in the blood plasma concentrations of melatonin in 4 adult Soay rams transferred from a period of long days (16L:8D) to one of constant darkness (0L:24D or DD). Day 0 is the last of the long days period. Each solid horizontal bar represents the duration of a significant peak for an individual ram. Periods of darkness are indicated by diagonal shading.

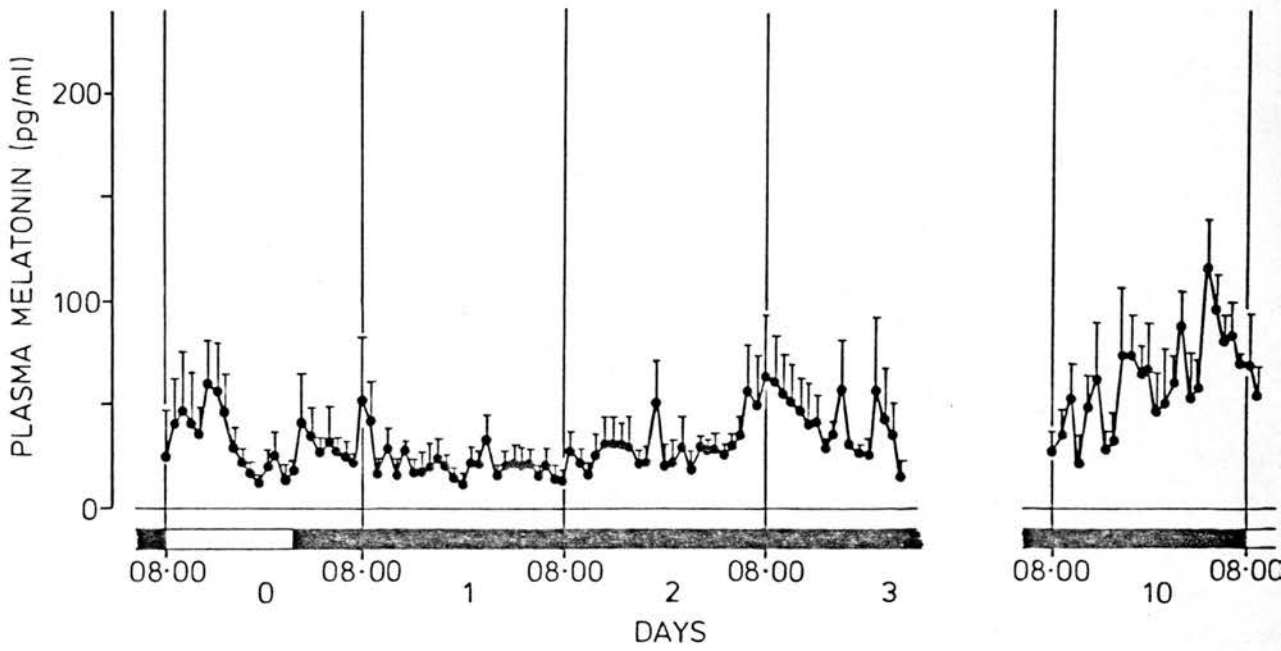


Fig. 6.10 Hourly changes in the concentration of melatonin (mean ± SEM) in the blood plasma of 4 adult SCGx Soay rams on Days 0, 1, 2 3 and 10 of an experiment in which the animals were transferr from a period of long days (16L:8D) to one of constant darkne (0L:24D or DD). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

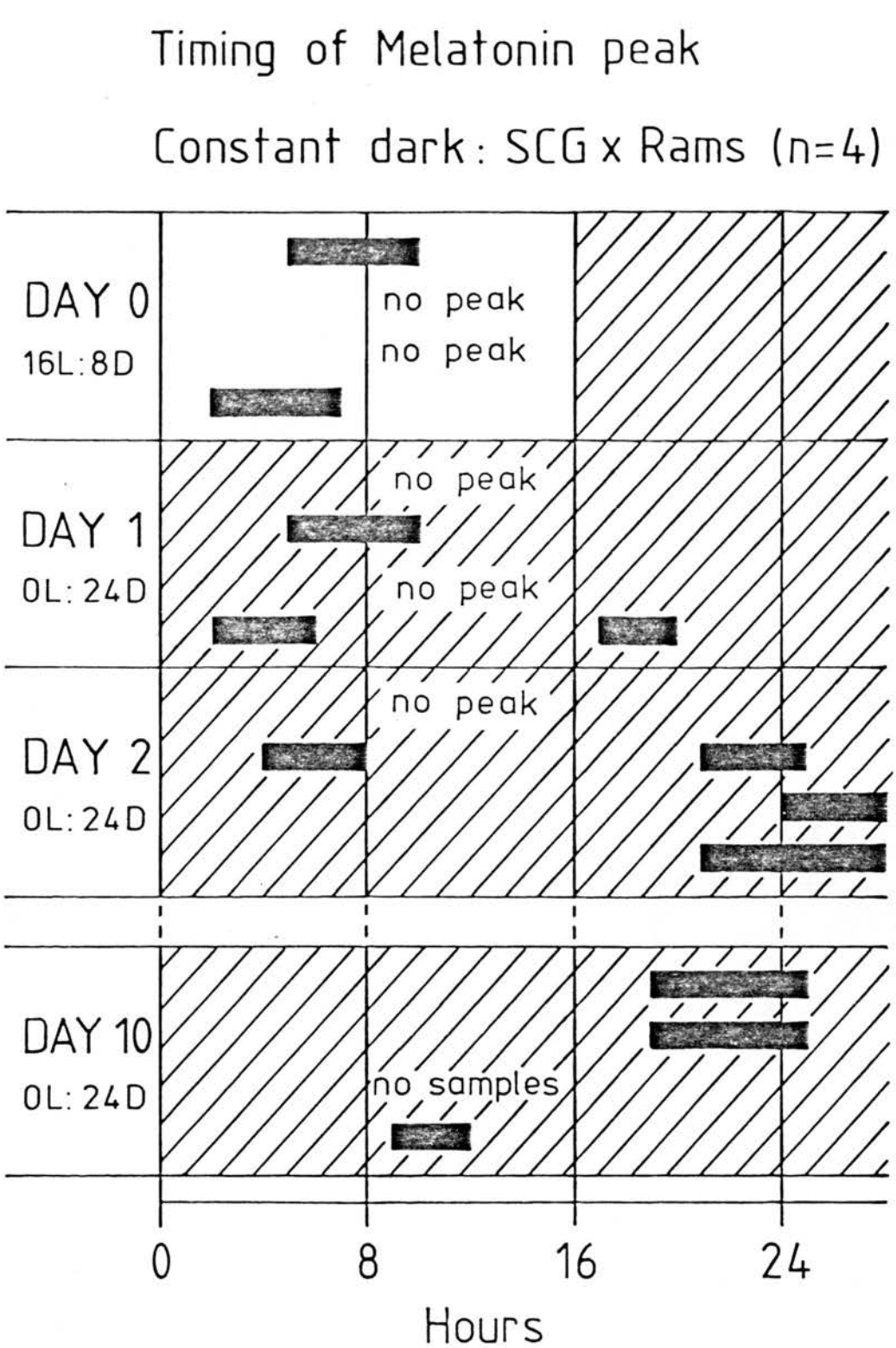


Fig. 6.11 Timing of peaks in the blood plasma concentrations of melatonin in 4 adult SCGx Soay rams transferred from a period of long days (16L:8D) to one of constant darkness (0L:24D or DD). Day 0 is the last of the long days period. Each solid horizontal bar represents the duration of a significant peak for an individual ram. Periods of darkness are indicated by diagonal shading.

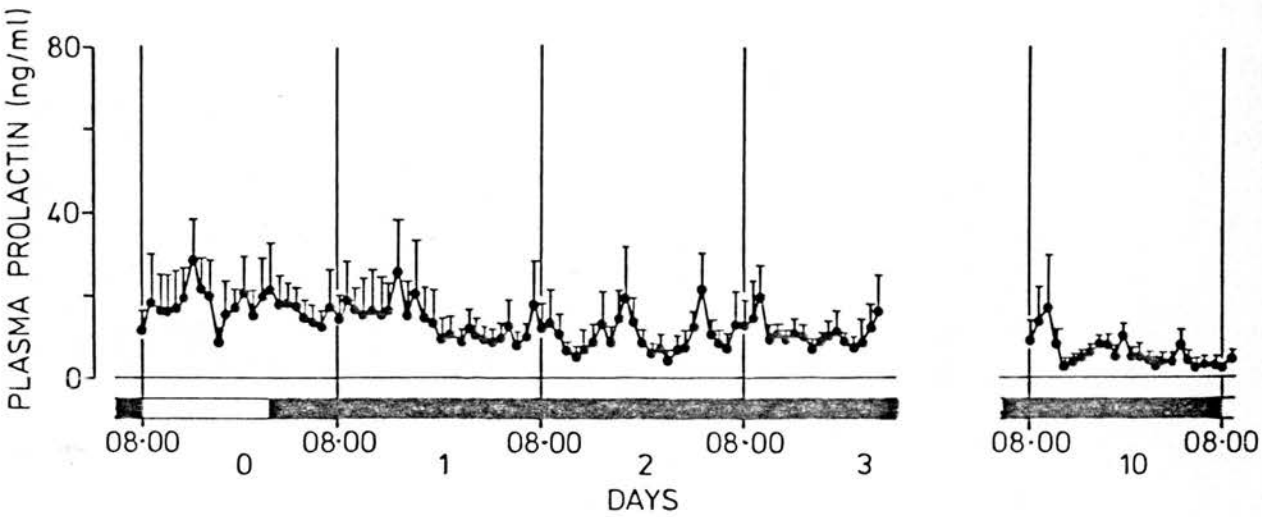


Fig. 6.12 Hourly changes in the concentrations of prolactin (mean ± SEM) in the blood plasma of 4 adult Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L:8D), to one of constant darkness (10L:24D or DD). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

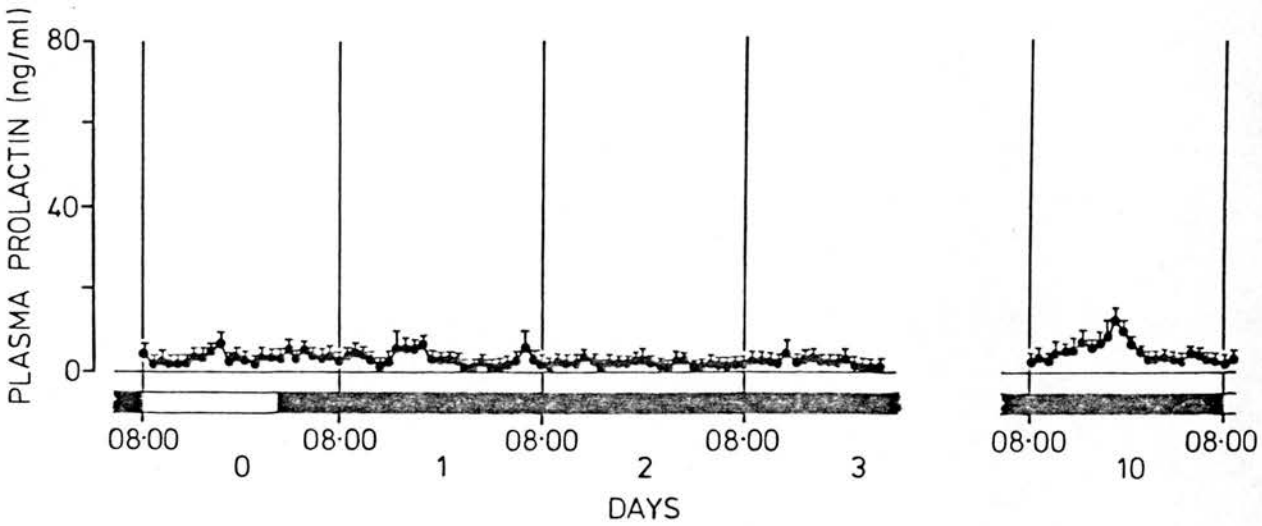


Fig. 6.13 Hourly changes in the concentration of prolactin (mean ± SEM) in the blood plasma of 4 adult SCGx Soay rams on Days 0, 1, 2, 3 and 10 of an experiment in which the animals were transferred from a period of long days (16L: 8D) to one of constant darkness (0L:24D or DD). Day 0 is the last day of the long days prior to the transfer. The timing of the periods of light (open bar) and darkness (closed bar) are shown at the bottom.

in the SCGx rams were significantly lower than those in the control rams. The hourly changes in the blood levels of PRL on days 0,1,2 and 10 of the treatment with LL are shown in Figs. 6.6 and 6.7.

6.3.2 Experiment 2: Exposure to DD for 10d

Blood MEL concentrations: The exposure of intact rams to 10d of DD resulted in an increase in the 24h mean blood concentrations of MEL, but these increments were not statistically significant (Table 6.1). There were also marked changes in the patterns of the blood levels of MEL (Fig. 6.8). On the last long day before the transfer to DD (day 0), the blood MEL peaks consistently occurred late in the day (14-24h), mainly during darkness, and this pattern was maintained on days 1 and 2 of the DD schedule (Fig.6.9). By day 10 of DD, however, these peaks were found to occur consistently earlier in the subjective day (9-24h, Fig. 6.9).

The exposure of the SCGx rams to 10d of DD resulted in some increases in the 24h mean blood levels of MEL, but these changes were not significant (Table 6.1; Fig. 6.10). During the last long day before DD, and during the period of DD, significant peak blood levels of MEL were found in the SCGx rams. However, these peaks did not occur consistently between the individual SCGx rams; furthermore, they occurred at irregular intervals during both the early and late portions of the subjective day (Fig. 6.11).

Blood PRL concentrations: In the control rams exposed to DD, there was a significant reduction in the 24h mean blood concentrations of PRL by day 10 (Table 6.2). In the SCGx rams exposed to this photoperiod, there was no significant change in the blood levels of PRL (Table 6.2). The hourly changes in the blood levels of PRL in the intact and SCGx rams on days 0,1,2 and 10 of DD are shown in Figs. 6.12 and 6.13.

6.4 DISCUSSION

The treatment of long day-accustomed intact Soay rams with 10d of either LL or DD did not cause a significant change in the 24h mean blood levels of MEL. This result therefore suggests that light and dark *per se* do not suppress or induce elevations in the circulating levels of MEL, and is similar to the data previously reported for two other species held under DD. Rhesus monkeys subjected to DD for 3d did not show significant increases in their mean daily CSF levels of MEL (Perlow, Reppert, Boyar & Klein, 1981). Similarly, 3d of DD did not cause a significant increase in the blood plasma levels of MEL in pony mares, although an increase was observed after 3 weeks of DD (Berglund, Sharp & Grubaugh, 1981).

In a similar experiment on the rhesus monkey, LL for 3d resulted in a suppression of the MEL concentrations in the CSF (Perlow *et al*, 1981). In an experiment in which sheep were provided with a pulse of light during a main period of darkness, Rollag, O'Callaghan & Niswender (1978) found that the blood plasma levels of MEL declined to daytime levels within 5-10 min of the onset of light. In contrast to the present results, the data of Perlow *et al* (1981) and Rollag *et al* (1978) thus imply that light has a direct suppressive effect upon MEL secretion. These discordant results are unlikely to be due to the assay of different body fluids since the changes in the CSF levels of MEL parallel those in the blood plasma (Rollag, Morgan & Niswender, 1977, 1978; Reppert, Perlow, Tamerkin & Klein, 1979). One possible explanation for these differing results may be that the intensity of the lights used in the present study may have been insufficient to suppress MEL secretion. Studies in both humans and rats have shown that the degree to which MEL secretion is suppressed is related to the

light intensity (Lewy, Wehr, Goodwin, Newsome & Markey, 1980a; Lewy, Wehr, Nurnberger, Becker, Rosenthal, Markey, Goodwin & Newsome, 1980b; Lynch, Rivest, Ronsheim & Wurtman, 1981).

In the present study, each of the treatments of the intact rams with LL or DD, was characterised by periods of consistently high and consistently low circulating levels of MEL (Figs. 6.3 & 6.9). The occurrence of these peak blood levels of MEL indicate the persistence of a 24h rhythm for at least the first 2d of the LL and DD treatments. It is not known, however, whether the 24h periodicity persisted up to day 10 of these treatments; for that information, hourly blood samples should have been collected for at least one more 24h period at the end of the LL and DD schedules.

Under DD, the control rams showed consistent changes in their blood patterns of MEL and by day 10 of DD they all showed an earlier occurrence of their peak blood levels of MEL. This observation could be taken to indicate a free-running of the rhythm in MEL secretion during DD; the shift in the timing of the MEL peak could be accounted for if the period of this free-running rhythm was less than 24h. The result obtained under LL differed from that obtained under DD, in that the timing of the occurrence of peak blood levels of MEL tended to remain the same as that under the previous treatment with long days. There is no clear explanation for why the LL and DD results differ. Nevertheless, both sets of data support the idea that the MEL rhythm represents an endogenously generated rhythm which is normally entrained by the external LD cycle. While the possible entraining effects of other external cues (e.g. daily time of feeding, fluctuations in ambient temperature) cannot be entirely excluded in the present study, the results from the DD experiment tend to argue against the influence

of such factors.

Previous studies in pony mares and rhesus monkeys have also shown the free-running characteristics of the MEL rhythm in the blood and CSF during DD (Berglund et al, 1981; Perlow et al, 1981). However, the only other study in which the free-running MEL rhythm was tested for under LL conditions (rhesus monkeys kept under LL for 3d: Perlow et al, 1981) showed a dampening, or loss, of this rhythm. As already pointed out, the intensity of light used might account for the different results obtained in the present experiment.

The blood levels of MEL in the SCGx rams remained relatively low during both the LL and DD exposures. Some SCGx rams showed significant peaks in their blood MEL concentrations; however, these peaks were poorly synchronised between individual SCGx rams, and they bore no relationship to the time of day. The low blood concentrations of MEL in the SCGx animals indicate that the sympathetic denervation of the pineal interfered with the secretion of MEL. The continued secretion of MEL in these rams suggests that the norepinephrine stimulus for MEL synthesis and secretion (see 1.2.1) may arise from elsewhere in the brain, or that there may be extra-pineal sources of MEL in sheep. Also, the disrupted patterns of MEL in the blood of the SCGx rams imply that the daily rhythm in MEL secretion is not generated from within the pineal itself.

During the 10d of exposure to both the LL and DD regimens, the control rams showed a significant reduction in their blood concentrations of PRL. Reduced blood PRL levels are normally associated with exposure to short daylengths (Lincoln, McNeilly & Cameron, 1978). The present LL results therefore suggest that this effect is not a simple response to a reduction in daylength (c.f. hour-

glass hypothesis; see Chapter 5), and that a circadian mechanism may determine the pattern of PRL secretion in response to changes in the photoperiod. Neither the LL nor the DD treatments resulted in any significant changes in the blood levels of PRL in the SCGx rams. It is therefore possible that the pineal somehow mediates the effects of the photoperiod upon PRL secretion.

CHAPTER 7

REPRODUCTIVE RESPONSES OF SOAY RAMS EXPOSED TO NATURAL PHOTOPERIODS AFTER ACTIVE IMMUNISATION AGAINST MELATONIN

7.1 INTRODUCTION

In order to investigate the specific role of MEL in the control of reproduction, active immunisation against this putative pineal hormone was carried out in three previous studies (golden hamster: Brown, Basinska, Bubenik, Sibony, Grota & Stancer, 1976; Knigge & Sheridan, 1976; laboratory rat: Niles, Brown & Grota, 1977). Despite the presence of antibodies capable of binding MEL, the reproductive responses of the rat and hamster were not affected. Nevertheless, in the present study, the same immunological approach was applied to test whether the photoperiodic control of reproduction in the Soay ram could be modified. Since the facilities to maintain the rams in a controlled environment were not available, this study was conducted on rams that were held out-of-doors.

7.2 MATERIALS AND METHODS

Animals: Twelve, 1-year-old Soay rams were used for these studies. During their first year, the rams have lived under natural daylengths on a farm near Cambridge (52°N). They were transferred to the farm of the Animal Breeding Research Organisation at Roslin, near Edinburgh (56°N) one month before the start of the experiment. The rams were held under natural daylengths, and allowed to graze freely throughout the study.

Immunogens: Two immunogens, referred to as the "Brown" and "ICI" preparations were used.

Brown immunogen: Initially, a conjugate of MEL-BSA prepared according to the formaldehyde condensation method of Grota & Brown (1974) was used. This material was provided by Dr. L.J. Grota (University of Rochester School of Medicine, N.Y.) through the courtesy of Professor G.M. Brown (Clarke Institute of Psychiatry, Toronto). The primary and

first 2 booster immunisations were made with this material, while the subsequent booster immunisations were made with a MEL-BSA conjugate prepared by the present author (all reagents, unless specified, obtained from Sigma Chemical Co., Poole, Dorset or BDH Chemicals, Poole, Dorset). The method followed for the synthesis of the MEL-BSA conjugate was based on that of Grota & Brown (1974) except that (1) tritiated MEL (200 x 10^3 dpm; New England Nuclear, GmBH) was added to the reaction mixture to allow calculation of the reaction yield (Lemaitre & Hartmann, 1980); (2) the conjugate was applied to a 125 x 2 cm column of Sephadex G-50 (Pharmacia), and eluted with 0.05M sodium phosphate buffer (pH 7.4) and the fractions containing protein and MEL-protein complexes (determined by UV spectrophotometery at 280 and 300 nm; Grota & Brown, 1974) were pooled; (3) the hapten-protein ratio was determined by measuring ^{3}H-MEL in the fractions of conjugate collected; and (4) the fractions of MEL-BSA conjugate were dialysed against several changes (20 l) of distilled water for 3 days and then against 0.05M sodium-phosphate buffer (pH 7.4) for 1 day. The hapten-to-protein molar ratio of the conjugate prepared in this way was found to be 6, a value comparable to that obtained by Grota & Brown (1974). The conjugate was lyophillised and stored at -20°C until required.

ICI immunogen: This immunogen was a conjugate of N-succinyl-5-methoxytryptamine and BSA. It was prepared and provided by Dr. H. Gregory (ICI Ltd., Cheshire).

Immunisation: The 12 rams were equally divided into 3 groups. The first group served as controls, receiving BSA as control immunogen; the second group was given the Brown immunogen and the third group received the ICI immunogen. One of the rams in this last group died during the study and was not replaced.

One primary and 4 booster immunisations were carried out during the course of 18 months. The primary immunisation was conducted on 30th May, 1980, when daylengths were increasing (Fig. 7.1). Booster doses were given 10,54,142 and 300 days later; the natural photoperiods on these occasions are approximately represented in Fig. 7.1.

For the primary immunisation, the rams received 100 µg of immunogen dissolved in 1.5 ml of 0.9% sterile saline (Travenol Laboratories, Norfolk) and emulsified in 1.5 ml of Freund's Complete Adjuvant (Difco Laboratories, West Molesey, Surrey), according to the method described by Fraser (1975). The emulsion was injected into 4 intra-dermal sites on the dorsal surface and 2 intra-muscular (thigh) sites of the rams, since injection into these sites has been reported to elicit a rapid production of antibodies to haptens (Vaitukaitis, Robbins, Nieschlag & Ross, 1971; Hurn, 1974).

Booster immunisations were made with 50 µg of immunogen dissolved in 1 ml of 0.9% sterile saline and emulsified with 1 ml Freund's Incomplete Adjuvant (Difco). The emulsion was injected into 4 dorsal intra-dermal sites.

Estimate of antibody production: At various intervals, blood was collected from the jugular veins of the rams by "Vacutainer", using heparin-free tubes to allow the harvesting of serum (see 2.3). The sera were stored at -20°C until assayed for their MEL content (see 2.5.1), and antibody titre as described below:-

To a 2.5 ml polystyrene test-tube (LP3, Sarstedt, Leicester), antibody was added at various dilutions (final dilutions between 1/1000 and 1/256,000) in 900 µl of 0.01M sodium phosphate buffer containing 0.1% gelatine (PBSG; pH7), followed by 20 pg of ^{3}H-MEL (New England Nuclear Chemicals, GmBH) in 100 µl PBSG. Similar tubes, from which

antibody was omitted, were set up to allow estimation of non-specific binding. The contents of the tubes were mixed thoroughly and incubated at 4°C for 36h. The antigen-antibody complex was precipitated by adding 1 ml saturated ammonium sulphate solution. The tube contents were mixed and then centrifuged at 2000xg for 20 minutes at 4°c. The supernatants were transferred to scintillation vials containing 10ml of 0.4% Butyl-PBD (Koch-Light, Colnbrook) in toluene (see 2.5.1) and counted in a β-spectrophotometer. The ^{3}H-MEL bound at each antiserum dilution was calculated, after taking into account non-specific binding. The dilution of antiserum that bound 50% of the labelled tracer (10pg ^{3}H-MEL) under these conditions was defined as the "titre" (Abraham, 1975).

Testicular activity: Changes in testicular activity were recorded at monthly intervals by measuring the size (diameter) of the testes (see 2.2).

On one occasion, approximately 11 months since the primary immunisation, when daylengths were increasing, the rams were bled at 20 minute intervals for a period of 8 hours. For this, cannulae were placed into the jugular veins of the rams during the previous 24h (see 2.3). The plasmas from the samples collected in this way were stored at -20°C until assayed for their testosterone concentrations according to the method described in 2.5.3.

This experiment was carried out in collaboration with Dr. G.A. Lincoln and has been partially described by Lincoln & Almeida (1982). The testosterone determinations were made by Mrs. R.A. Cunningham.

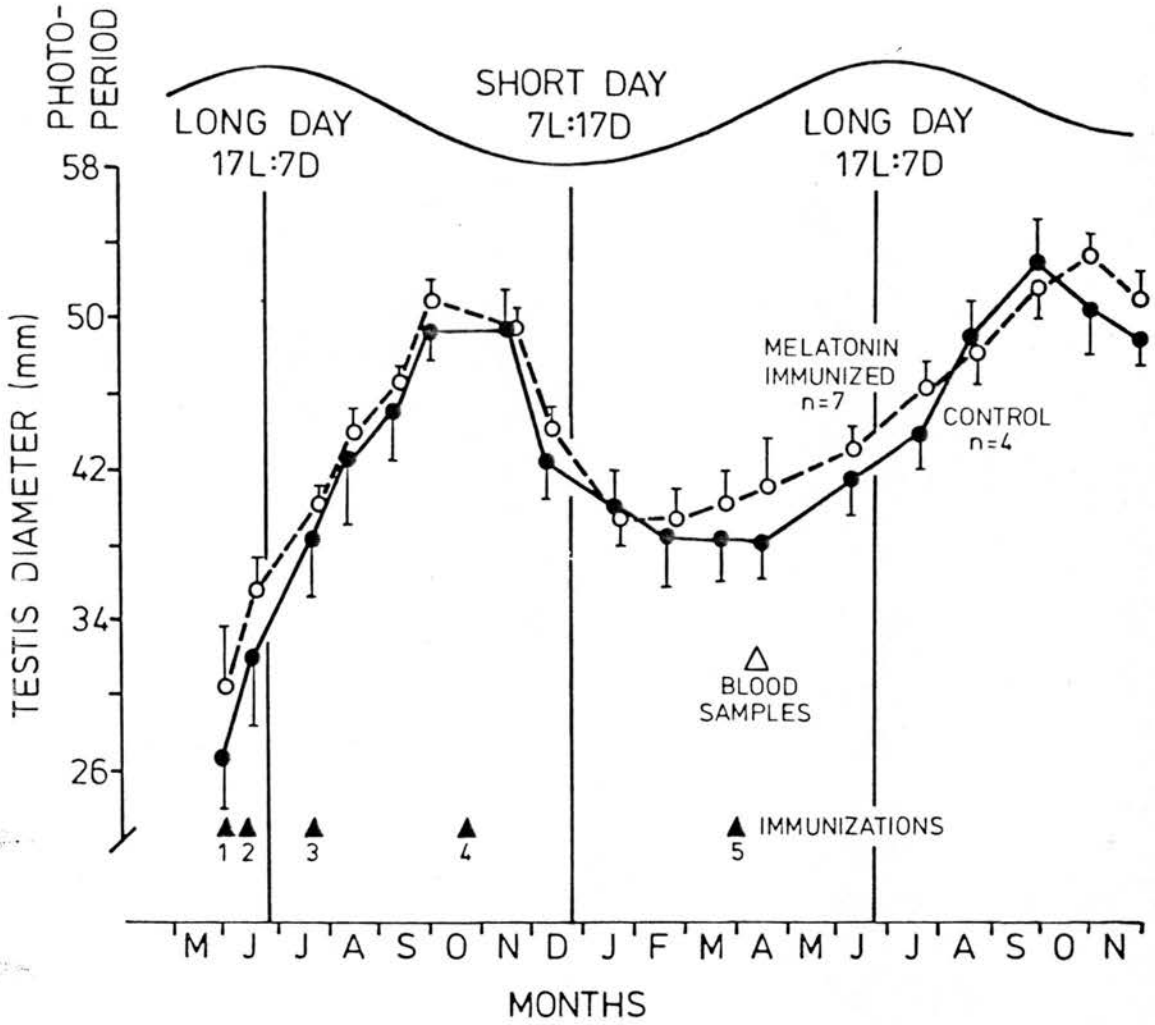


Fig. 7.1 Changes in the size of the testes of 11 Soay rams exposed to natural photoperiods for 18 months. 4 of the rams (controls) were immunized against bovine serum albumin (BSA) while the other 7 rams were immunized against a conjugate of melatonin and BSA on the occasions indicated by the filled arrow-heads. On the occasion marked by the open arrow-head, blood samples were collected from the rams at 20 minute intervals for 8 hours. The plasmas obtained were assayed for their content of testosterone, and those results are shown in Fig. 7.2.

7.3 RESULTS

7.3.1 Antibody titres and MEL concentrations: The Brown and ICI conjugates both elicited immune responses in the rams and antibody titres ranging between 1/1000 and 1/128,000 were recorded. The highest titres were found about 2 weeks after the booster injections. The blood of the rams with high antibody titres contained high blood levels of MEL during daylight hours, and there was a significant correlation between antibody titres and circulating levels of MEL ($r=0.77$, $p < 0.001$). The specificity of the antisera were not tested.

The sera from those rams injected with BSA as immunogen (control group) did not bind ^{3}H-MEL. The daytime concentrations of MEL in these samples were significantly lower than those found in the samples from the immunised rams (control rams: 8.4 $\pm$ 1.3 pg/ml; immunised rams: 114.4 $\pm$ 12.9 pg/ml; means $\pm$ SEM, based on samples collected on 8 occasions).

7.3.2 Reproductive changes: The changes in the size of the testes of the two immunised groups of rams did not differ significantly. They were thus combined for comparison with the changes found in the control rams (Fig. 7.1). At the start of the experiment (May), the testes of both the control and immunised rams were regressed. During the summer months (June-September) there was a marked increase in testicular diameter, and peak testicular sizes were observed during the autumn months (October-November). Subsequently, the testes of both groups of rams regressed to reach a nadir during the winter months (December-March). Testicular growth was resumed during the following spring and summer months, and maximum testicular sizes were again recorded during October and November. The seasonal reproductive cycle of the immunised rams did not differ from that of the control rams.

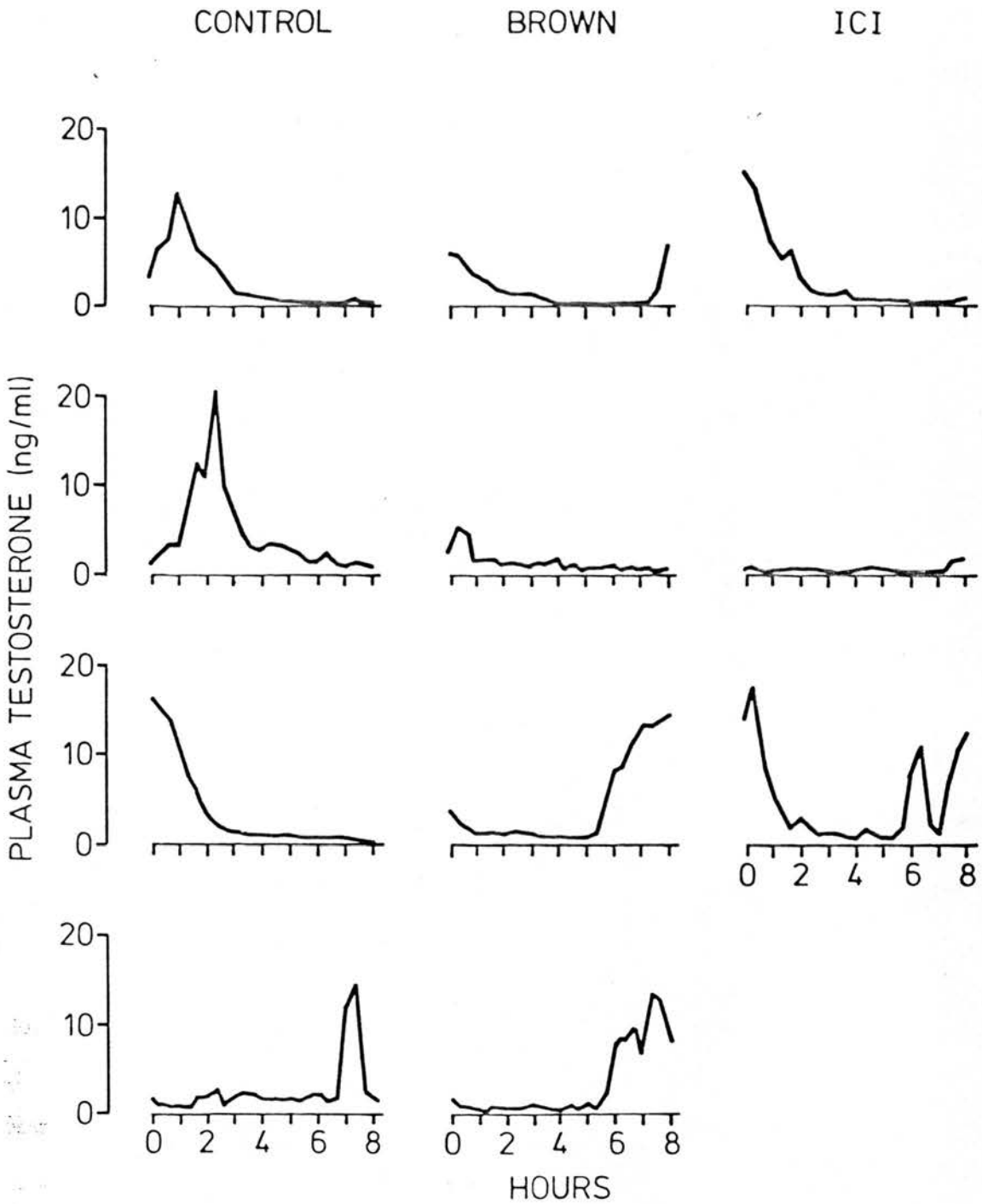


Fig. 7.2 Patterns of testosterone secretion in individual Soay rams exposed to natural photoperiods during the course of immunizat against BSA (controls) or melatonin (Brown and ICI). The samples were collected at 20 minute intervals for 8 hours 2 weeks after a booster immunization (see Fig. 7.1) and when daylengths were beginning to increase. No significant differences were found between mean plasma concentrations of testosterone or number of episodes of testosterone release between the control and immunized rams.

When blood plasma samples were collected at 20 minute intervals in April, shortly after the 5th and final booster immunisation, and when all the animals were beginning to redevelop their testes, the blood plasma levels of testosterone were found to be similar in the immunised and control animals; this included the mean plasma concentrations of testosterone, as well as the minimum and maximum concentrations and the number of episodic releases of testosterone (Fig. 7.2).

7.4 DISCUSSION

Antibody titres ranging between 1/1000 and 1/128000 were found in the Soay rams which had been actively immunised against conjugates of MEL-BSA and N-succinyl-5-methoxytryptamine-BSA. These rams also had high circulating levels of MEL during daylight hours, suggesting that the antibodies were effective in binding large amounts of endogenous MEL. This result is different from that of Niles, Brown, Pang & Grota (1979) who found that rats immunised against MEL had undetectable levels of the hormone. Since the pineals of their immunised rats contained large amounts of MEL, those authors concluded that the MEL antibodies prevented the secretion of the hormone. The MEL content of the pineals of the sheep used in the present study was not assayed; however, the elevated levels of MEL in their blood suggests that the immunisation had not totally blocked the secretion of MEL.

The presence of antibodies against MEL did not interfere with the rams' seasonal reproductive cycles (Fig. 7.1). Previous studies in which laboratory rats and golden hamsters were immunised against MEL, also failed to find any changes in reproduction (Brown *et al*, 1976; Knigge & Sheridan, 1976; Niles *et al*, 1977; Brown, Grota, Bubenik & Tsui, 1981). The present data for rams indicate that MEL concentrations may not be causally related to the control of seasonal

reproductive cycles. However, before any conclusions can be drawn from such studies, it is necessary to demonstrate the efficacy of the immunisation. For this, a number of factors need to be considered, e.g. the specificity of the antisera, the effects of immunisation on the daily blood patterns of MEL, and the ability of the antibodies to neutralise the effects of MEL in the brain.

The specificities of the antisera raised in the present study were not tested. However, the high titres of antiserum capable of binding ^{3}H-MEL, together with the high day-time blood levels of MEL, point to the fact that the antisera raised had a high capacity for MEL. While the present antisera are not presumed to be 100% specific for MEL immuno-neutralisation of other closely related indoleamines would not have detracted from the main result.

Another related point is that in previous chapters it was found that there is a daily rhythm in the secretion of MEL, and that the daily blood patterns of MEL correlated with changes in reproductive status. Since no night-time samples were collected in this study, it is not possible to know whether this rhythm persisted during the period of immunisation. If it did, it is possible that the rams still experienced a normal rhythm in MEL secretion which was dictated by the photoperiod; also, without knowing what the free circulating levels of MEL were, this cannot be determined.

The final point is that the site of action of MEL is still equivocal. Receptors for MEL have been found in the medial-basal hypothalamus and several peripheral tissues, including the gonads (Cardinali, 1981; Cardinali & Vacas, 1981). If MEL acts upon the brain, the failure of the immunisation against MEL to affect reproduction might reflect the inability of the antibodies to cross the

blood-brain barrier (c.f. Bradbury, 1979). This point could be clarified by studying the reproductive effects of the passive transfer of MEL antibodies directly into the cerebrospinal fluid (c.f. de Wied, 1979; Sakuma & Pfaff, 1980).

While the use of active immunisation against a specific hormone at first appears to be a powerful method for investigating the action of that hormone (Nieshlag, 1975; Edwards & Johnson, 1976; Hillier & Cameron, 1976), there are considerable difficulties associated with the interpretation of the results, as illustrated by the present study. The present experiment would have been improved if the rams had been exposed to controlled changes in the photoperiod, so that specific effects of the photoperiod upon reproduction could be observed. Another approach would be to passively transfer antibodies to MEL into rams at specific times during their exposure to a controlled photoperiod, and to test whether the photoperiodic stimulus could be modified by the antiserum.

CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

Breeding activity in most temperature-zone mammals is confined to a brief period each year. Since the period of gestation is usually of a fixed length, seasonal breeding ensures that the offspring arrive at a time of year when climatic and nutritional factors are most likely to enhance the chances of the survival of the young and of the lactating mother. Seasonal breeding involves a seasonal reactivation of the hypothalamic-pituitary gonad (HPG) axis, and the signal for this reactivation is provided by the external environment. In fact, the environment provides several cues which have the potential to synchronize reproductive activity with the prevailing seasons, e.g. temperature, precipitation, daylength and nutrition. Most mammals appear to have adapted to respond to changes in the daily photoperiod since this factor is the most stable in the long-term. In sheep, both decreasing natural daylengths and artificial short daylengths trigger the reactivation of the HPG axis (Lincoln & Short, 1980).

Theoretically, changes in daylength can be measured by one of two mechanisms. In the first, the animal counts the number of hours of light or darkness, or the ratio of light to dark. In this "hour-glass model", daylength is then reflected by the accumulation of the products of some photochemical reaction (Hillman, 1979; Rusak & Zucker, 1979). The second mechanism involves the animal's generation of endogenous 24h (circadian) rhythms, and the way in which the LD cycle entrains these rhythms, (Pittendrigh & Minis, 1964; Bünning, 1973; Rusak & Zucker, 1979). In most birds and mammals in which photoperiodic time measurement has been investigated, it appears that the circadian system is involved (Follett, 1978; Rusak & Zucker, 1979). Using a "resonance photoperiod" experimental protocol (Nanda & Hamner, 1958), it was shown

that a circadian mechanism most probably underlies the photoperiodic regulation of testicular activity in the Soay ram (Chapter 5).

The neural structures involved in the generation and entrainment of circadian rhythms in mammals have only recently been identified. Most circadian rhythms are now believed to originate from the bilateral suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Rusak & Zucker, 1979). These rhythms are entrained by the external 24h LD cycle to have periods of precisely 24h; photic information reaches the SCN by way of a direct retinohypothalamic tract (Moore & Klein, 1974; Legau & Winans, 1981). The efferent connections of the SCN with the pineal gland are particularly well known. The SCN-pineal neural pathway is a tortuous one, involving the superior cervical ganglia (SCG), as described earlier (1.1.3). The normal daily variations in pineal activity are disrupted by interruption of this neural pathway (Moore, 1978). In all mammalian species investigated, including the Soay sheep (Chapter 3), there is evidence for diurnal changes in the patterns of MEL secretion. These changes are related to the time of day, with the highest concentrations being found during darkness and the lowest concentrations during periods of light. In the present study it was shown that SCGx in the Soay ram results in a disruption of the daily pattern in the blood plasma levels of MEL (Chapter 3).

Two types of experiment carried out in this project indicated that the daily variation in the blood patterns of MEL in the Soay ram might represent a circadian rhythm. Firstly, when rams were exposed to 10d of either constant light (LL) or constant darkness (DD), there was evidence of the persistence of an approximately 24h rhythm in the blood levels of MEL, i.e, the MEL rhythm was apparently able to free-run in the absence of entraining LD cues (Chapter 6). Secondly, exposure

of rams to two resonance photoperiods of 8L:40D and 8L:28D showed that only the 8L:40D regimen was capable of entraining and maintaining a 24h pattern in the blood concentrations of MEL (Chapter 5).

As already indicated, the blood patterns of MEL normally closely reflect the proportions of light and dark in each LD cycle. Evidence for this was provided in Chapter 3, when it was shown that the blood patterns of MEL differ between long days of 16L:8D and short days of 8L:16D. These different patterns of MEL secretion can be partly explained in terms of differential entrainment by the two photoperiods. While differences were found in the phases of MEL secretion during long days and short days, differences were also found in the amplitude of MEL secretion under these two photoperiods. The night-time concentrations of MEL were greater during the 16L:8D regimen than during the 8L:16D regimen, suggesting that this may be a separate effect of the photoperiods; whether these amplitude differences are an example of external coincidence (see p.157), or whether they simply reflect limitations of the synthetic capacity of the pineal, is not clear from the data available. Nevertheless, these changing patterns of MEL secretion may be taken as an example of how changes in the photoperiod are translated and transformed into humoral messages. It is through such a mechanism that MEL is hypothesised to have a role in the control of seasonal reproduction.

There is ample evidence which implicates MEL in the control of mammalian seasonal reproductive cycles (see 1.4). Changes in the photoperiod which normally stimulate gonadal recrudescence or regression, apparently become ineffective after PINX or SCGx. The daily patterns of MEL secretion are abolished or disrupted in PINX and SCGx animals; however, replacement therapy with MEL in such animals can

re-create some of the reproductive effects of the photoperiod. MEL, when administered to intact mammals causes gonadal regression in some instances and recrudescence in others; factors such as the prevailing photoperiod, reproductive status and mode of administration appear to determine the reproductive effects of exogenous MEL (Goldman, Hall, Hollister, Roychoudhury, Tamarkin & Westrom, 1979; Turek & Campbell, 1979; Hoffmann, 1981b,c). The ability of MEL to recreate or negate the effects of the photoperiod therefore suggests that it might be involved in conveying changes in the LD cycle to the neuroendocrine-gonadal axis.

The data collected in the present study show several correlations between the blood patterns of MEL and the photoperiod-mediated changes in the testicular activity of Soay rams. Firstly, intact Soay rams, when held under alternating 16 week cycles of long days and short days, showed cycles of testicular regression and redevelopment; these cycles were correlated with consistent changes in their blood patterns of MEL which reflected the daily photoperiod (Chapter 3). In contrast, SCGx rams failed to show testicular responses to the changes in photoperiod, and their blood patterns of MEL did not correspond to the LD cycle. Secondly, during two resonance photoperiod schedules of 8L:40D and 8L:28D, only those rams kept under the 8L:40D regimen redeveloped their testes in a normal fashion. Those rams kept under the 8L:28D regimen redeveloped their testes sluggishly, and with much individual variation (Chapter 5, c.f. Chapter 3). The blood patterns of MEL also differed between the two groups. While peak blood levels of MEL were consistently found late in the day, at 24h intervals in the 8L:40D group, peak blood levels of MEL occurred at irregular intervals in the individual rams in the 8L:28D group. Thirdly, prolonged exposure of

Soay rams to either long days or short days resulted in the development of photorefractoriness, the onset of which was marked by a disruption in the daily rhythm in the blood concentrations of MEL (Chapter 4).

On their own, the above correlations in the sheep do not prove a causal relationship between MEL and reproduction. However, such a relationship is suggested by the extensive experimental data available for smaller mammals (Turek & Campbell, 1979; Reiter, 1980; Hoffmann, 1981a,b,c; Cardinali, 1981). The correlations drawn from the present data mainly refer to patterns of MEL secretion related to the time of day, rather than to the absolute blood levels of the hormone. A correlation between blood MEL concentrations and reproduction existed only in the first case cited. Since the SCGx rams had relatively low blood levels of MEL and maintained large testes throughout the study (Chapter 3), MEL might be considered to have a simple inhibitory effect upon the HPG axis; otherwise, the data imply that it is the time of day at which MEL occurs that is crucial in determining the reproductive response of Soay rams, i.e. the role of MEL may be to translate the photoperiod. How then might MEL convey information about the photoperiod to the HPG axis?

Changes in daylength are known to alter the secretion of the gonadotrophins and thus the activity of the gonads. Detailed studies of the photoperiodic regulation of gonadotrophin secretion in the Soay ram indicate that these events are the result of changes in the frequency of the episodic release of GnRH by the hypothalamus (Lincoln & Short, 1980). It is therefore logical to suppose that the action of MEL might be to modify the activity of the hypothalamic GnRH neurones. Indeed, specific high-affinity receptors for MEL have been located in the medial basal hypothalmus (MBH), an area rich in GnRH-secreting

cells (Cardinali, Vacas & Boyer, 1979; Cardinali, 1981; Cardinali & Vacas, 1981). There are several ways in which MEL reaching the MBH could alter GnRH release. For example, MEL is known to alter the electrical activity of nerve cells (Demaine & Kann, 1979; 1981), and may therefore be envisaged to act either directly upon the noradrenergic GnRH neurones or indirectly upon the neighbouring dopaminergic and serotoninergic neurones; an interaction between these monoaminergic systems of the brain is believed to be involved in the control of GnRH secretion (McCann, 1981). Intracellular mechanisms of action have also been proposed for MEL. The first proposal is based on the observation that MEL inhibits prostaglandin synthesis and release (Cardinali, 1981); prostaglandins have been suggested to serve as the intracellular "second messengers" for the stimulatory effects of norepinephrine upon GnRH synthesis and secretion (McCann, 1981). The second is that MEL influences the rate of axonal transport of GnRH, since the hormone affects the rate of microtubule synthesis (Cardinali, 1981). In view of the above, it is interesting that evidence obtained in the golden hamster suggests that the pineal gland may be involved in mediating photoperiod-induced changes in hypothalamic-pituitary responsivity to testosterone feedback (Turek, 1979b).

A simple hypothetical model for the way in which MEL might influence seasonal breeding in sheep would be one in which MEL acted to inhibit GnRH secretion. In that model, the degree of GnRH inhibition would vary according to the prevailing photoperiod, since the patterns of MEL secretion are known to reflect the proportions of light and dark in each LD cycle. However, the present studies in sheep indicate that there is no simple relationship between the amounts of MEL in the blood and reproductive activity. Instead, a good correlation was found

between the temporal patterns in the blood levels of MEL and reproductive activity. Do these results then imply that MEL inhibits reproduction, but that this inhibitory effects is manifest at only certain times of the day?

There is good experimental evidence that the reproductive responses of the golden hamster and the ferret to exogenous MEL are dependent upon the time of day at which the hormone is administered (Tamarkin, Westrom, Hamill & Goldman, 1976; Herbert, 1981). For example, MEL given during the late afternoon of a long day causes gonadal involution in golden hamsters, whereas the same dose given earlier in the day has no inhibitory effects upon reproduction (Tamarkin et al, 1976; 1977).

On the basis of the foregoing data, is is possible to construct a model for the role of MEL in the photoperiodic control of reproduction. In this model, the role of the photoperiod is to dictate rhythms in MEL secretion and in brain responsivity to MEL. These two rhythms are entrained in different ways in response to changes in the photoperiod, and it is the phase relationships between these rhythms that determine the photoperiodic response of the gonads. Thus, when MEL secretion occurs at the same time as a period of responsiveness to MEL, reproduction is inhibited. This idea fits into the "internal coincidence model" which will be discussed later.

There is some evidence which suggests that MEL itself might influence rhythms in hypothalmic activity. Firstly, Yates & Herbert (1976), working with ferrets, showed that timed injections of MEL during long days could result in short day patterns in the hypothalamic content of serotonin. Secondly, while SCGx Soay rams continue to show 24h rhythms in their blood levels of PRL, the timing of these rhythms,

relative to the time of day, is different from that observed in control rams (Lincoln, 1979a,b). These results may be caused by the direct effects of MEL upon rhythms in brain monoamine activity, or by its effects upon the SCN. Indeed, there is evidence to implicate an action of MEL upon the SCN. For example, the SCN have been shown to bind MEL (Brown, Grota, Bubenik, Niles & Tsui, 1981), the reproductive effects of MEL implants are greatest when placed in the region of the SCN (Glass & Lynch, 1981; 1982), and the reproductive effects of MEL differ between SCN-lesioned and SCN-intact animals (Rusak & Morin, 1976; Bittman, Goldman & Zucker, 1979, Rusak, 1980).

The appropriate phasing of various circadian rhythms which results in a photoperiodic response forms the basis of the "internal coincidence model" which Pittendrigh & Minis (1964) proposed as an extension to Bünning's original circadian hypothesis. In Bünning's model, now referred to as the "external coincidence model", the photoperiod has a dual role: to entrain the circadian oscillations and to drive the photoperiodic response by a temporal coincidence with a photo-inducible phase (Saunders, 1977). In contrast, in the "internal coincidence model" the photoperiod serves a single purpose: to entrain one or more circadian oscillators.

In the present study, an attempt was made to investigate whether the "internal coincidence model" might apply in sheep. In the "resonance photoperiod" experiment (Chapter 5), it was hoped to demonstrate that particular phase relationships between rhythms in MEL secretion and hypothalamic activity might be responsible for determining the photoperiodic response. As a measure of hypothalamic activity, the blood concentrations of PRL were measured. However, since the 8L:40D schedule was interpreted as short days might be, the

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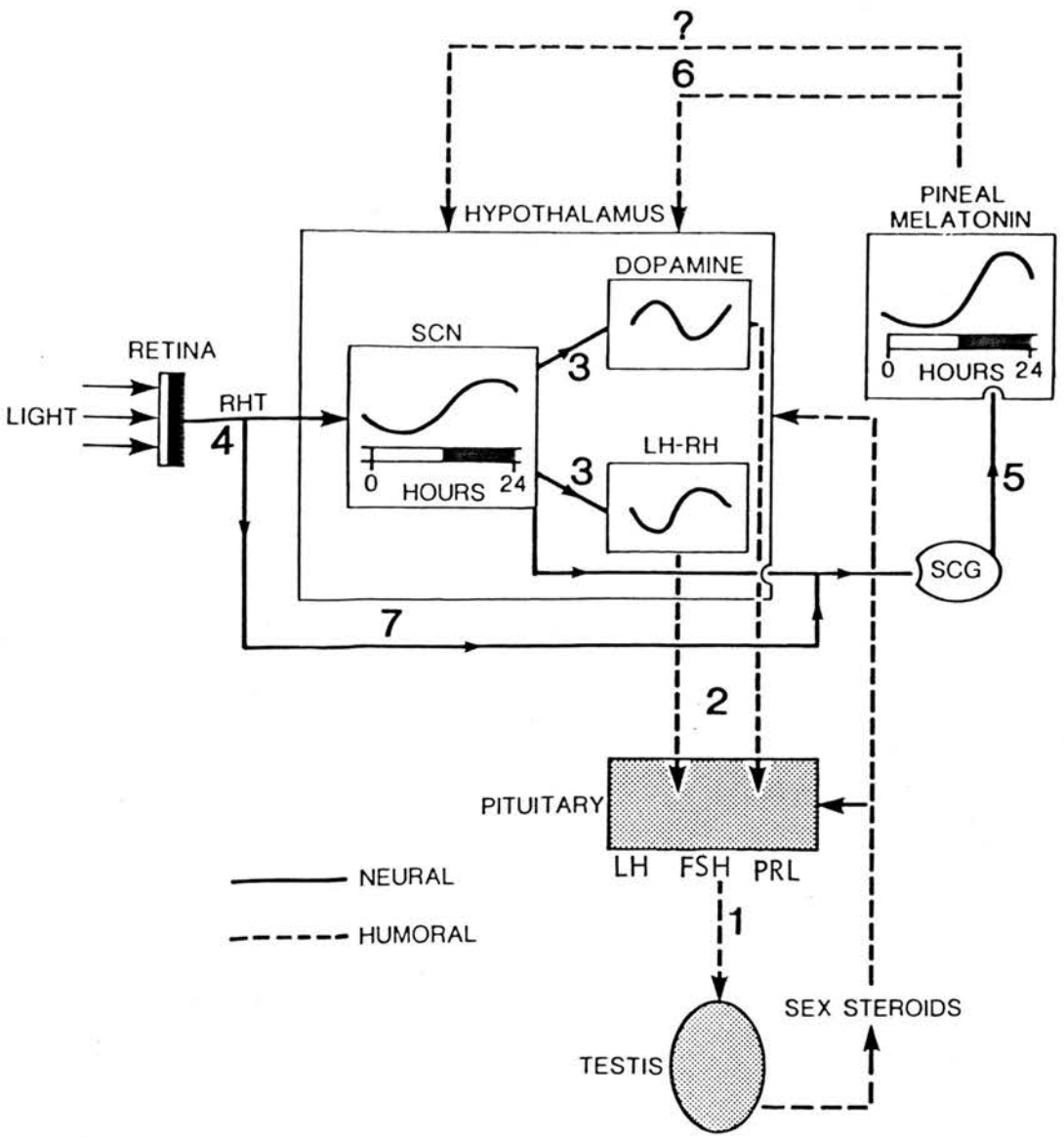

Fig. 8.1

blood plasma levels of PRL were so markedly reduced that a rhythm could not be detected. The blood plasma electrolyte concentrations were also investigated since they would be expected to reflect 24h rhythms in feeding and mineral metabolism. However, it appears that these latter rhythms do not depend directly upon the external LD cycle for their entrainment, and so these measures provided no information on whether the "internal coincidence model" applies to the sheep. For this, other rhythms would need to be studied in detail.

Although the internal and external coincidence hypotheses form two distinct models, there is no obvious reason why they should both not operate in causing the photoperiodic response. While most of the present data favour the internal coincidence model, it should be remembered that different amounts of MEL are secreted during long days and short days (Chapter 3), suggesting the involvement of an external coincidence model. Furthermore, the quantitative effects of light in suppressing MEL secretion are well documented in several mammalian species, including the sheep (Rollag, O'Callaghan & Niswender, 1978; Lewy, Wehr, Goodwin, Newsome & Markey, 1980; Lynch, Rivest, Ronsheim & Wurtman, 1981; Perlow, Reppert, Boyar, & Klein, 1981).

A simple representation of the structures and interactions proposed to be involved in the photoperiodic control of testicular activity in the ram is shown in Fig. 8.1. In this plan, the release of LH, FSH and PRL from the anterior pituitary regulates the activity of the testes (1). The secretion of the gonadotrophins and PRL are controlled by the release of GnRH and dopamine (DA) from the hypothalamus (2). The secretions of the endocrine hypothalamus are induced to show 24h rhythms by the SCN (3). The 24h rhythms which originate in the SCN are endogenously generated, but their phases may be entrained by the external LD cycle; photic information reaches the

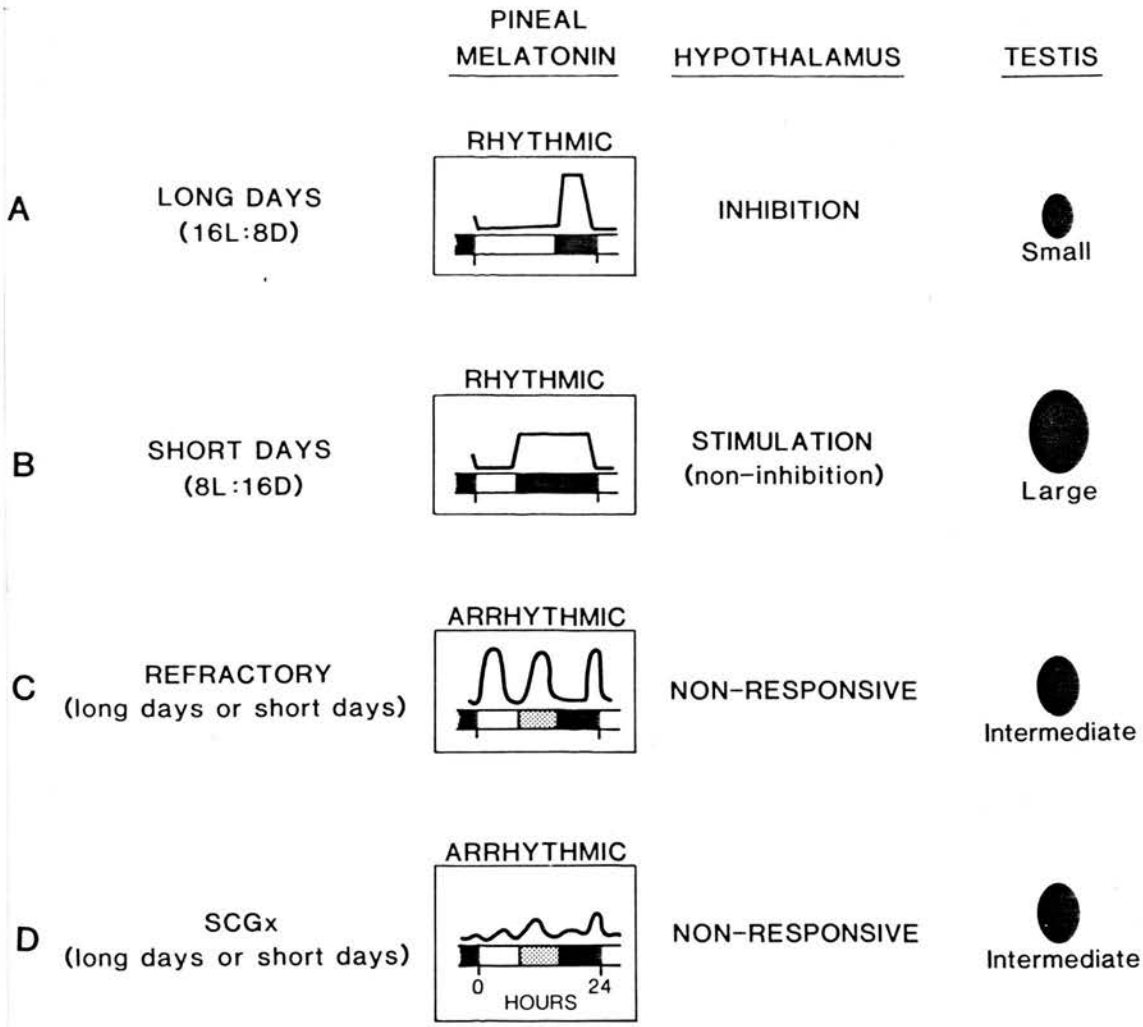
PINEAL MELATONIN
HYPOTHALAMUS
TESTIS
A
LONG DAYS (16L:8D)
RHYTHMIC
INHIBITION
Small
B
SHORT DAYS (8L:16D)
RHYTHMIC
STIMULATION (non-inhibition)
Large
C
REFRACTORY (long days or short days)
ARRHYTHMIC
NON-RESPONSIVE
Intermediate
D
SCGx (long days or short days)
ARRHYTHMIC
0
24
HOURS
NON-RESPONSIVE
Intermediate

Fig. 8.2

SCN via the RHT (4). The SCN are also connected to the pineal gland via the SCG, and regulate the activity of the pineal (5). The pineal produces and releases MEL according to a 24h rhythm which reflects changes in the LD cycle. This MEL may feed back to regulate the activity of the hypothalamic neurosecretory cells or of the SCN (6). It is also possible that changes in the LD cycle can regulate MEL secretion by a pathway which does not include the SCN (7).

Some of the results obtained in the present study on Soay rams can be explained in terms of the model proposed above. For example, during long days, MEL secretion is restricted to a short phase of darkness, GnRH secretion is inhibited and the testes regress (Fig. 8.2a). Assuming that MEL acts to suppress GnRH release if it occurs at a time when the hypothalamus is responsive to the effects of MEL, it may be envisaged that during long days, there is a coincidence between the occurrence of MEL and the phase of hypothalamic responsivity to MEL. Under short days, MEL is again secreted mainly during darkness, but it is now apparently ineffective in suppressing reproductive activity (Fig. 8.2b). This result may be explained in terms of non-coincidence, due to the rhythms being entrained differently by short days compared to long days. After prolonged exposure to either long days or short days, the daily rhythm in MEL secretion is disrupted by unknown mechanisms, and the testes appear to be only partially stimulated (Fig. 8.2c). Similarly, disrupted MEL rhythms and a partial stimulation of the gonads is observed in SCGx rams exposed to either long days or short days (Fig. 8.2d). These intermediate responses of the testes may be explained in terms of partial coincidence due to the arrhythmicity in MEL secretion and, or, the loss of rhythmicity in hypothalamic

responsivity to MEL. It should be noted that the refractory and SCGx states are not necessarily equivalent; for example, the SCGx rams had much lower circulating levels of MEL than the intact photorefractory rams.

The simple model described above, and shown in Fig. 8.2, presumes the occurrence of internal coincidence. It does not, however, preclude the simulataneous occurrence of external coincidence; after all, the amplitude and duration of MEL secretion differs between sheep exposed to long days and short days. The model also assumes that the brain's responsiveness to MEL is restricted to one particular time of day. It is however possible that the brain is continuously responsive to MEL, except for a short specific daily period during which it is non-responsive. The model supposes a single, GnRH-inhibiting, action for MEL. This supposition is based upon electrophysiological and biochemical studies (Demaine & Kann, 1979; 1981; Cardinali, 1981) and studies involving implants of MEL (Glass & Lynch, 1981; 1982; Lincoln & Almeida, 1982). The data showing that MEL has pro-gonadal effects tends to be inconsistent, and could be interpreted as the failure of MEL to inhibit reproduction. In the context of the proposed model, such an interpretation is supported by data indicating that MEL may participate in the phase-setting of various brain rhythms (Yates & Herbert, 1976; Lincoln, 1979a,b; Rusak & Zucker, 1979).

Lastly, the model proposed above is based entirely on the involvement of a circadian mechanism. It should be noted that data now exsists for the sheep, that an hour-glass model might equally apply. For example, it is possible to terminate anoestrous in ewes under long days by simply increasing the duration of their exposure to MEL, by the administration of MEL either by feeding (intact ewes: D.J. Kennaway,

T.A. Gilmore & R.F. Seamark, personal communication) or by infusion (PINX ewes: E.L. Bittman, personal communication).

It is clear from the present results that the mechanisms underlying the photoperiodic control of seasonal breeding in the sheep are complex. The present study has contributed to the understanding of these mechanisms by demonstrating correlations between the patterns of MEL in the blood plasma and specific photoperiod-mediated reproductive conditions. It provides a framework upon which future studies, aimed at elucidating the role of MEL in the control of seasonal breeding in the rams, can be based.

REFERENCES

ABRAHAM, G.E. (1975): Characterization of anti-steroid antisera. In: Steroid Immunoassay. Fifth Tenovus Workshop. E.H.D. Cameron, S.G. Hillier and K. Griffiths. Eds: Alpha-Omega Press, Cardiff p.67-78.

APPLETON, A.B. and WAITES, G.M.H. (1975): A surgical approach to the superior cervical ganglion and related structures in the sheep. J. Physiol., (Lond). 135, 52-57.

ARENDT, J. (1978): Melatonin assays in body fluids. J. Neural Transm. Suppl. 13, 265-278.

ARENDT, J. (1979): Radioimmunoassayable melatonin: circulating patterns in Man and sheep. Prog. Brain Res. 52, 249-257.

ARENDT, J. and SYMONS, A.M. (1981): Discussion. In: Biological Clocks in Seasonal Reproductive Cycles. B.K. Follett and D.E. Follet. Eds: Wright, Bristol pp. 275-276.

ARENDT, J., SYMONS, A.M. and LAUD, C. (1981): Pineal function in the sheep: evidence for a possible mechanism mediating seasonal reproductive activity. Experentia, 37, 584-589.

ARENDT, J., WETTERBERG, L., HEYDEN, T., SIZONENKO, P.C. and PAUNIER, L., (1977): Radioimmunoassay of melatonin: human serum. Horm. Res. 8, 65-75.

AXELROD, J., (1978): Introductory remarks on regulation of pineal indoleamine synthesis. J. Neural Transm. 13, 73-79.

BACON, A., SATTLER, C. and MARTIN, J.E. (1981): Melatonin effect on the hamster pituitary response to LHRH. Biol. Reprod. 24, 993-999.

BAKER, J.R., (1938): The evolution of breeding seasons. In: "Evolution: essays on aspects of evolutionary biology". Ed: de Beer, G.R. pp. 161-177. Oxford University Press, London.

BAKER, J.R. and ANSON, R.M. (1932): Factors affecting the breeding of the field mouse (Microtus agrestis)I. Light. Proceedings of the Royal Society B, 110, 313-322.

BALEMANS, M.G.M., (1979): Indole metabolism in the pineal gland of the rat; some regulatory aspects. Prog. Brain Res. 52, 221-228.

BITTMAN, E.L. (1978c): Melatonin prevents refractoriness to short days in male hamsters. Proc. Soc. Exp. Biol, Med. 158, 359-362.

BITTMAN, E.L., GOLDMAN, B.D. and ZUCKER, I. (1979): Testicular responses to melatonin are altered by lesions of the suprachiasmatic nuclei in golden hamsters. Biol. Reprod. 21, 647-656.

BITTMAN, E.L. and ZUCKER, I. (1981): Photoperiodic termination of hamster refractoriness: participation of the pineal gland. Biol. Reprod. 24, 568-572.

BLAIR-WEST, J.R., COGHLAN, J.P., DENTON, D.A., GODING, J.R., WINTOUR, M. and WRIGHT, R.D. (1963): The control of aldosterone secretion. Rec. Prog. Horm. Res. 19, 311-383.

BOLTON, A.E., and HUNTER, W.M. (1973): A new method for labelling protein hormones with radioiodine for use in radioimmunoassay. J. Endocrinol. 55, xxx

BRACKMANN, M., (1977): Melatonin delays puberty in the Djungarian hamster. Naturwissenschaften, 64, 642-643.

BRACKMANN, M. and HOFFMANN, K. (1977): Pinealectomy and photoperiod influence testicular development in the Djungarian hamster. Naturewissenschaften, 64, 341-342.

BRADBURY, M. (1979): Concept of the blood-brain barrier. Wiley, Chichester, pp. 465.

BROWN, G.M., BASINSKA, J., BUBENIK, G., SIBONY, D., GROTA, L.J. and STANCER, H.C. (1976): Gonadal effects of pinealectomy and immunization against N-acetylindolealkylamines in the hamster. Neuroendocrinology, 22, 289-297.

BROWN, G.M., GROTA, L.J., BUBENIK, G., NILES, L.J. and TSUI, H. (1981): Physiologic regulation of melatonin. In: Melatonin: Current status and perspectives. Eds. N. Birau and W. Schloot, Pergamon Press, Oxford, p. 95-112.

BROWNSTEIN, M. and AXELROD, J. (1974): Pineal gland: 24 hour rhythm in norepinephrine turnover. Science, 184, 163-165.

BALEMANS, M.G.M., SMITH, I. and de REUVER, G.F. (1981): Changes in the circadian rhythmicity of N- and O-acetyltransferase activities in the pineal gland of 38 day old male Wistar rats when examined under white, red and green light. J. Neural Transm. 52, 83-93.

BARRELL, G.K. and LAPWOOD, K.R. (1978/79): Effects of modifying olfactory and pineal gland function on the seasonality of semen production and plasma luteinizing hormone, testosterone and prolactin levels in rams. Anim. Reprod. Sci. 1, 229-243.

BARRELL, G.K. and LAPWOOD, K.R. (1979): Effects of pinealectomy on the secretion of luteinizing hormone, testosterone and prolactin in rams exposed to various lighting regimes. J. Endocrinol. 80, 394-405.

BENSON, B., LARSEN, B.R. and FINDELL, P.R. (1981): Melatonin and other pineal products. In: Melatonin: Current Status and Perspectives. N. Biran and W. Schloot, Eds. Pergamon Press, Oxford, pp. 55-64.

BERGLUND, L.A., SHARP, D.C. and GRUBAUGH, W. (1981): Effects of constant darkness on melatonin rhythms in pony mares. Abstr. 97, 14th Society for the Study of Reproduction Meeting.

BINKLEY, S., 1980): Functions of the pineal gland. In: Avian Endocrinology. Eds: A. Epple and M.H. Stetson, Academic Press, New York. p.53-74.

BINKLEY, S., MACBRIDE, S.E., KLEIN, D.C. and RALPH, C.L. (1975): Regulation of pineal rhythms in chickens: refractory period and non-visual light perception. Endocrinology, 96, 848-853.

BIRAU, N. and SCHOOT, W. (1981): Melatonin: Current Status and Perspectives. Pergamon Press, Oxford.

BITTMAN, E.L. (1978a): Hamster refractoriness: the role of insensitivity of pineal target tissues. Science, 202, 648-650.

BITTMAN, E.L. (1978b): Photoperiodic influences on testicular regression in the golden hamster: termination of scoto-refractoriness. Biol. Reprod. 18, 871-877.

BUNNING, E. (1973): The Physiological Clock. 3rd English Edition. Springer Verlag, Berlin.

BUTLER, J.E. (1975): The immune response to protein and protein-conjugated antigens and the possible consequences of resultant in vivo reactions. In: Immunization with hormones in reproduction research. Ed: E. Nieschlag, North Holland Publishing Co. Amsterdam.

CAMPBELL, R.N. (1974): St. Kilda and its sheep. In: Island Survivors: The Ecology of the Soay Sheep of St. Kilda. Eds: P.A. Jewell, C. Milner and J. Morton-Boyd. The Athlone Press, London, pp. 8-35.

CARDINALI, D.P. (1981): Melatonin. A mammalian pineal hormone. Endocrine Rev. 2, 327-346.

CARDINALI, D.P., LYNCH, H.J. and WURTMAN, R.J. (1972): Binding of melatonin to human and rat plasma proteins. Endocrinology, 91, 1213-1218.

CARDINALI, D.P. and VACAS, M.I. (1981): Molecular endocrinology of melatonin: receptor sites in brain and peripheral organs. In: Melatonin: Current status and perspectives. Eds: N. Birau and W. Schloot, Pargamon Press, Oxford, 237-246.

CARDINALI, D.P., VACAS, M.I. and BOYER, E.E. (1979): Specific binding of melatonin in bovine brain. Endocrinology, 105, 437-441.

CARDINALI, D.P., VACAS, M.I. and GEJMAN, P.V. (1981): The sympathetic superior cervical ganglia as peripheral neuro-endocrine centers. J. Neural Transm. 52, 1-21.

CARDINALI, D.P., and WURTMAN, R.J. (1975): Control of melatonin synthesis in the pineal organ. In: Frontiers in Pineal Physiology. Ed: M.D. Altschule, The MIT Press, Cambridge, Mass. pp. 12-14.

CHANG, N., EBELS, I and BENSON, B. (1979): Preliminary characterization of bovine pineal prolactin releasing (PPRF) and relase inhibiting factor (PPIF) activity. J. Neural Transm. 46, 139-151.

CLARKE, J.R. (1981): Physiological problems of seasonal breeding in eutherian mammals. In: Oxford Reviews of Reproductive Biology. C.A. Finn, Ed. Clarendon Press, Oxford, pp. 242-312.

COLE, H.H. (1953): Problems in the field of physiology of reproduction of farm animals. Iowa St. Coll. J. Sci. 28 , 133.

COLLIN, J-P. (1971): Differentiation and regression of the cells of the sensory line in the epiphysis cerebri. In: The Pineal Gland. G.E.W. Wolstenholme and J. Knight, Eds. Churchill-Livingstone, Edinburgh, pp. 79-125.

CORKER, C.S. and DAVIDSON, D.W. (1978): A radioimmunoassay for testosterone in various biological fluids without chromatography. J. Steroid Biochem. 9, 373-374.

DAFNY, N., (1980): Photic input to rat pineal gland conveyed by both sympathetic and central afferents. J. Neural Transm. 48, 203-208.

DEGUCHI, T. (1975): Shift in circadian rhythm of serotonin: acetyl coenzyme A N-acetyltransferase activity in pineal gland of rat in continuous darkness or in the blinded rat. J. Neurochem. 25, 91-93.

DEGUCHI, T. (1979): A circadian oscillator in cultured cells of chicken pineal gland. Nature, 282, 94-96.

DEGUCHI, T. (1981): Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature 290, 706-707.

DEMAINE, C. and KAHNN, H.C. (1979): Modification of the electrical activity of hypothalamic neurones by pineal indoles. Prog. Brain Res. 52, 373-375.

DEMAINE, C. and KANN, H.C. (1981): Effect of pinealectomy in the electrical responses of hypothalamic neurones to the application of melatonin. In: Melatonin, Current Status and Perspectives. N. Birau and W. Schloot, Eds. Pergamin Press, Oxford. pp. 123-128.

DOGTEROM J., SNIJDEWINT, F.G.M. PEVET, P. and BUIJS, R.M. (1979): On the presence of neuropeptides in the mammalian pineal gland and subcommissural organ. Prog. Brain Res. 52, 465-470.

DOMANSKI, E., PRZEKOP, F. and POLKOWSKA, J. (1980): Hypothalamic centres involved in the control of gonadotrophin secretion. J. Reprod. Fert. 58, 493-499.

DUBE, D., LE CLERC, R. and PELLETIER, G. (1975): Immunohistochemical detection of growth hormone inhibiting hormone (SRIF) in guinea pig brain. Cell Tissue Res. 161, 385.

DUCKER, M.J., BOWMAN, J.C. and TEMPLE, A. (1973): The effects of constant photoperiod on the expression of oestrus in the ewe. J. Reprod. Fert. Suppl. 9 143-150.

EDINGER, T. (1956): Paired pineal organs. Progress in Neurobiology 1, 120-129.

EDWARDS, R.G. and JOHNSON, M.H. (1976): Physiological effects of immunity against reproductive hormones. Cambridge University Press, Cambridge, pp. 283.

ELLIOTT, J.A. (1981): Circadian rhythms, entrainment and photoperiodism in the Syrian hamster. In: Biological Clocks in Seasonal Reproductive Cycles. B.K. Follett and D.E. Follett, Eds. Wright, Bristol. pp. 203-217.

ELLIOTT, J.A. and GOLDMAN, B.D. (1981): Seasonal reproduction: photoperiodism and biolgical clocks. In: Neuroendocrinology of Reproduction. N.T. Adler, Ed. Plenum Press, N.Y. pp. 377-423.

ELLIOTT, J.A., STETSON, M.H. and MENAKER, M. (1972): Regulation of testis function in golden hamsters: a circadian clock measures photoperiodic time. Science, 178, 771-773.

ELLIS, G.B., LOSEE, S.H. and TUREX, F.W. (1981): Pinealectomy abolishes the short photoperiod induced attenuated castration response in male golden hamsters. In: Pineal Function. C.D. Matthews and R.F. Seamark, Eds. Elsevier/North Holland Biomedical Press, Amsterdam pp. 87-94.

FARRAR, G.M. and CLARKE, J.R. (1976): Effect of chemical sympathectomy and pinealectomy upon the gonads of voles (Microtus agrestis) exposed to short photoperiods. Neuroendocrinology, 22, 134-143.

FLINT, A.P.F., RENFREE, M.B. and WEIR, B.J. (1981): Embryonic diapause in mammals. J. Reprod. Fert. Suppl. 29.

FOLLETT, B.K. (1973): Circadian rhythms and photoperiodic time measurement in birds. J. Reprod. Fert. Suppl. 19, 5-18.

FOLLETT, B.K. (1978): Photoperiodism and seasonal breeding in birds and mammals. In: "Control of Ovulation". pp. 267-293. Eds: D.B. Crighton, G.R. Foxcroft, N.B. Haynes and G.E. Lamming, Butterworths, London.

FOLLETT, B.K., MATTOCKS, P.W. Jr. and FARNER, D.S. (1974): Circadian function in the photoperiodic induction of gonadotropin secretion in the white-crowned sparrow, (Zonotrichia leucophrys gambelii). Proc. Natl. Acad. Sci., USA., 71, 1666-1669.

FOLLETT, B.K., ROBINSON, J.E., SIMPSON, S.M. and HARLOW, C.R. (1981): Photoperiodic time measurement and gonadotrophin secretion in quail. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett, Wright, Bristol, pp. 185-201.

FOLLETT, B.K. and SHARP, P.J. (1969): Circadian rhythmicity in photoperiodically induced gonadotrophin release and gonadal growth in the quail. Nature, 223 , 968-971.

FRASER, H.M. (1975): Inhibition of luteinizing hormone-releasing hormone by antibodies. Ph.D. Thesis, University of Dundee.

FRASER DARLING, F., (1974): Foreword. In: Island Survivors: The Ecology of the Soay Sheep of St. Kilda. Eds: P.A. Jewell, C. Milner and J. Morton-Boyd, pp. xi-xii.

GASTON, S., and MENAKER, M. (1967): Photoperiodic control of hamster testis. Science, 158, 925-928.

GLASS, J.D. and LYNCH, G. R. (1981): Melatonin: identification of sites of antigonadal action in mouse brain. Science, 214, 821-823.

GLASS, J.D. and LYNCH, G.R. (1982): Evidence for a brain site of melatonin action in the white-footed mouse, (Peromyscus leucopus). Neuroendocrinology, 34, 1-6.

GOLDMAN, B., HALL, V., HOLLISTER, C., REPPERT, S., ROYCHOUDHURY, P., YELLON, S. and TAMARKIN, L. (1981): Diurnal changes in pineal melatonin content in four rodent species: relationship to species. Abstr. 63rd Annual Meeting, Endocrine Society, Cincinnati, July, 1981.

GOLDMAN, B.D., HALL, V., HOLLISTER, C., ROYCHOUDHURY, P., TAMARKIN, L. and WESTROM, W. (1979): Effects of melatonin on the reproductive system in intact and pinealectomized male hamsters maintained under various photoperiods. Endocrinology, 104, 82-88.

GOODMAN, R.L. and KARSCH, F.J. (1981): The hypothalamic pulse generator: a key determinant of reproductive cycles in sheep. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett, Wright, Bristol, pp. 223-236.

GROCOCK, C.A. and CLARKE, J.R. (1974): Photoperiodic control of testis activity in the vole (Microtus agrestis). J. Reprod. Fertil. 39, 337-347.

GROTA, L.J. and BROWN, G.M. (1974): Antibodies to indolealkylamines: Serotonin and Melatonin. Can. J. Biochem. 52, 196-202.

GROTA, L.J., HOLLOWAY, W. and BROWN, G.M. (1981): Melatonin receptors in pineal. In: Melatonin: Current status and perspectives. N. Birau and W. Schloot, Eds. Pergamon Press, Oxford. pp. 257-261.

GRUBB, P. (1974): The rut and behaviour of soay rams. In: Island Survivors: The Ecology of the Soay sheep of St. Kilda, Eds: P.A. Jewell, C. Milner and J. Morton Boyd, The Althlone Press, London, pp. 195-223.

GRUBB, P. and JEWELL, P.A. (1973): The rut and the occurrence of oestrus in the soay sheep of St. Kilda. J. Reprod. Fert. Suppl. 19, pp. 491-502.

GWINNER, E. (1981): Circannual rhythms: their dependence on the circadian system. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett. Wright, Bristol pp. 153-169.

HAFEZ, E.S.E. (1952): Studies on the breeding season and reproduction of the ewe. J. Agric. Sci. 42, 189-265.

HAMASKI, D.I. and EDER, D.J. (1977): Adaptive radiation of the pineal system. In: The Visual system in Vertebrades. F. Crescitelli, Ed. Springer-Verlag, Berlin, pp. 497-535.

HAMNER, W.H. (1963): Diurnal rhythm and photoperiodism in testicular recrudescence of the house finch. Science, 142, 1294- 1295 .

HAMNER, W.H. (1964): Circadian control of photoperiodism in the house finch demonstrated by interrupted-night experiments. Nature, 203, 1400-1401.

HAMNER, W.H. (1968): The photorefractory period of the house finch. Ecology, 49, 211-227.

HARLOW, H.J., PHILLIPS, J.A. and RALPH, C.L. (1981): Day-night rhythms in plasma melatonin in a mammal lacking a distinct pineal gland, the nine-banded armadillo. Gen. Comp. Endocrinol. 45, 212-218.

HERBERT, J. (1969): The pineal gland and light-induced oestrus in ferrets. J. Endocrinol. 43, 625-636.

HERBERT, J. (1971): The role of the pineal gland in the control by light of the reproductive cycle of the ferret. In: The Pineal Gland. Eds: G.E.W. Wolstenholme and J. Knight. Churchill Livingstone, Edinburgh. pp. 303-327.

HERBERT, J. (1972): Initial observations on pinealectomized ferrets kept for long periods in either daylight or artificial illumination. J. Endocrinol. 55, 591-597.

HERBERT, J. (1981): The pineal gland and photoperiodic control of the ferret's reproductive cycle. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett. Wright, Bristol. pp. 261-276.

HERBERT, J., STACEY, P.M. and THORPE, D.H. (1978): Recurrent breeding seasons in pinealectomized or optic-nerve-sectioned ferrets. J. Endocrinol. 78, 389-397.

HEUBNER, O. (1898): Tumor der glandula pinealis. Dtsch. med. Wscher. 24, 214-

HILLIER, S.G. and CAMERON, E.H.D. (1976): Physiological effects of immunity against steroid hormones in the rat. In: Physiolgical Effects of Immunity Against Reproductive Hormones. Eds: R.G. Edwards and M.H. Johnson. Cambridge University Press, Cambridge p.91-120.

HILLMAN, W.S. (1979): Photoperiodism in Plants and Animals. No. 107. Carolina Biology Readers (Ed: J.J. Head) Burlington, North Carolina.

HOFFMANN, K. (1973): The influence of photoperiod and melatonin on testis size, body weight and pelage colour in the Djungarian hamster (Phodopus sungorus). J. Comp. Physiol. 85, 267-282.

HOFFMAN, K. (1974): Testicular involution in short photoperiods inhibited by melatonin. Naturwissenschaften, 61, 364-365.

HOFFMAN, K. (1978): Effects of short photoperiods on puberty, growth and moult in the Djungarian hamster (Phodopus sungorus). J. Reprod. Fert. 54, 29-35.

HOFFMAN, K. (1979): Photoperiod, pineal, melatonin and reproduction in hamsters. Prog. Brain Res. 52, 397-414.

HOFFMANN, K. (1981a): Photoperiodism in Vertebrates. In: Handbook of Behavioural Neurobiology Vol. 4. Ed: J. Aschoff, Plenum Publishing Corp. N.Y. pp. 449-473.

HOFFMANN, K. (1981b): The role of the pineal gland in the photoperiodic control of seasonal cycles in hamsters. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett, Wright, Bristol pp. 237-250.

HOFFMANN, K. (1981c): Photoperiodic function of the mammalian pineal organ. In: The Pineal Organ: Photobiology-Biochemometery-Endocrinology. Eds: A. Oksche and P. Pevet, Elsevier/North Holland Biomedical Press, Amsterdam. pp. 123-138.

HOFFMANN, K., ILLNEROVA, H., and VANECEK, J. (1981): Effect of photoperiod and of one-minute light at night-time on the pineal rhythm on N-acetyltransferase activity in the Djungarian hamster (Phodupus sungorus). Gen. Comp. Endocrinol. 24, 551-556.

HOFFMANN, K. and KUDERLING, I. (1975): Pinealectomy inhibits stimulation of testicular development by long photoperiods in a hamster (Phodopus sungorus). Experentia, 31, 122-123.

HOFFMANN, K. and KUDERLING, I. (1977): Antigonadal effects of melatonin in pinealectomized Djungarian hamsters (Phodopus sungorus). Naturwissenschaften, 64, 339-340.

HOFFMAN, R.A. and REITER, R.J. (1966): Responses of some endocrine organs of female hamsters to pinealectomy and light. Life Science, 5, 1147.

HOWLES, C.M., WEBSTER, G.M. and HAYNES, N.B. (1980): The effect of rearing under a long or short photoperiod on testes growth,plasma testosterone and prolactin concentrations, and the development of sexual behaviour in rams. J. Reprod. Fertil. 60, 437-447.

HUNTER, W.M. (1974): Preparation and assessment of radioactive tracers. Br. Med. Bull. 30, 18-23.

HUNTER, W.M. (1978): Radioimmunoassay. In: Handbook of Experimental Immunology. Ed: D.M. Weir. Blackwell Scientific Publications, Oxford, 14.1-14.40.

HUNTER, W.M., NARS, P.W. and RUTHERFORD, F.J. (1975): In: Steroid Immunoassay: 5th Tenovus International Workshop. Eds: E.H.D. Cameron, J.G. Hillier and K. Griffiths. Alpha-Omega Publishing Ltd. Cardiff. pp. 141-164.

HURN, B.A.L. (1974): Practical problems in raising antisera. Br. Med. Bull. 30, 26-28.

ILLNEROVA, H. and VANECEK, J. (1980): Pineal rhythm in N-acetyltransferase activity in rats under different artificial photoperiods and in natural daylight in the course of a year. Neuroendocrinology, 31, 321-326.

JEWELL, P.A. and GRUBB, P. (1974): The breeding cycle, the onset of oestrus and conception in Soay sheep. In: Island Survivors: The Ecology of the Soay Sheep of St. Kilda. Eds: P.A. Jewell, C. Milner and J. Morton-Boyd. The Athlone Press, London pp. 224-241.

JEWELL, P.A., MILNER, C. and MORTON-BOYD, J. (1974): Island Survivors: The Ecology of the Soay Sheep of St. Kilda. The Athlone Press, London. 386p.

JOHNSTON, P.G. and ZUCKER, I. (1980a): Photoperiodic regulation of the testes of adult white-footed mice (Peromyscus leucopus). Biol. Reprod. 23, 859-866.

JOHNSTON, P.G. and ZUCKER, I. (1980b): Photoperiodic regulation of the testes of adult white-footed mice (Peromyscus leucopus). Biol. Reprod. 23, 859-866.

JOHNSTON, P.G. and ZUCKER, I. (1980c): Photoperiodic regulation of reproductive development in white-footed mice (Peromyscus leucopus). Biol. Reprod. 22, 983-989.

KAO, L.W.L. and WELSZ, J. (1977): Release of gonadotrophin releasing hormone (GnRH) from isolated, perifused medial-basal hypothalamus by melatonin. Endocrinology, 100, 1723-1726.

KAPPERS, J.A. (1965): Survey of the innervation of the epiphysis cerebri and the accessory pineal organs of vertebrates. Prog. Brain Res. 10, 87-153.

KAPPERS, J.A. (1977): The Pineal Organ: An Introduction. In: The Pineal Gland. Eds: G.E.W. Wolstenholme and J. Knight. Churchill-Livingstone, Edinburgh, pp. 3-34.

KAPPERS, J.A. (1976): The mammalian pineal gland, a survey. Acta neurochir. 34, 109-149.

KAPPERS, J.A. (1979): Short history of pineal discovery and research. Prog. Brain Res. 52, 3-22.

KARSCH, F.J. and FOSTER, D.L. (1981): Environmental control of seasonal breeding: a common final mechanism governing seasonal breeding and sexual maturation. In: Environmental Factors in Mammal Reproduction. Eds: D. Gilmore and B. Cook. MacMillan, London pp. 30-53.

KENNAWAY, D.J., FRITH, R.G., PHILLIPOU, G., MATTHEWS, E.D. and SEAMARK, R.F. (1977): A specific radioimmunoassay for melatonin in biological tissue and fluids and its validation by gas chromatography-mass spectrometetery. Endocrinology, 101, 119-127.

KENNAWAY, D.J., OBST, J.M., DUNSTAN, E.A. and FRIESEN, H.G. (1981): Ultradian and seasonal rhythms in plasma gonadotrophins, prolactin, cortisol and testosterone in pinealectomized rams. Endocrinology, 108, 639-646.

KING, J.A. and MILLAR, R.P. (1981): Decapeptide luteinizing hormone releasing hormone in ovine pineal gland. J. Endocrinol. 91, 405-414.

KITAY, J.I. and ALTSCHULE, M.D. (1954). The Pineal Gland. Harvard University Press, Cambridge, Mass. 280 pp.

KLEIN, D.C. (1978): The pineal gland: a model of neuro-endocrine regulation. In: The Hypothalamus. Eds: S. Reichlin, R.J. Baldersarini and J.B. Martin. Raven Press, New York. p.303-327.

KLEIN, D.C. and WELLER, J.L. (1970): Indole metabolism in the pineal gland: A circadian rhythm in N-acetyltransferase. Science, 169, 1093-1095.

KLEIN, D.C., WELLER, J., REPPERT, S., TAMARKIN, L., and AUERBARCH, D. (1979): Molecular events involved in circadian rhythms in the pineal gland. In: Biological Rhythms and their Central Mechanism. Eds: M. Suda, O. Hayaishi and H. Nakagarva. Elsevier/North Holland Biomedical, Tokyo. 147-148.

KNIGGE, K.M., and SHERIDAN, M.N. (1976): Pineal function in hamsters bearing melatonin antibodies. Life Sci. 19, 1235-1238.

KRIEGER, D.T. (1979): Rhythms in CRF, ACTH and cortico-steroids. In: Endocrine Rhythms. Ed: D.T. Krieger, Raven Press, N.Y. pp. 123-147.

LAMMING, G.E., MOSELEY, S.R. and McNEILLY, J.R. (1974): Prolactin release in sheep. J. Reprod. Fert. 40, 151-168.

LAUD, C.A. and SMITH, I. (1979): The binding of metho-tyindoles to human plasma proteins. Prog. Brain Res. 52, 513-515.

LEES, A.D. (1966): Photoperiodic timing mechanisms in insects. Nature, 210, 986-989.

LEES, A.D., and HARDIE, J. (1981): The photoperiodic control of polymorphisn in aphids: neuroendocrine and endocrine components. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett. Wright, Bristol, pp. 125-135.

LEGAN, S.J. and WINANS, S.S. (1981): The photoneuroendocrine control of seasonal breeding in the ewe. Gen. Comp. Endocrinol. 45, 317-328.

LEMAITRE, B.J. and HARTMANN, L. (1980): Preparation of anti-melatonin antibodies and antigenic properties of the molecule. J. Immunol. Methods. 32, 339-347.

LEONE, R.M., SILMAN, R.E., HOOPER, R.J.L., FINNIE, M.D.A. CARTER, S.J., EDWARDS, R., SMITH, I., TOWELL, P., and MULLEN, P.E. (1979): A sensitive and specific assay for 5-methoxytryptophiol in plasma. J. Endocrinol. 82, 243-257.

LERNER, A.B., CASE, J.D. and HEINZELMAN, R.V. (1959): Structure of melatonin. J. Amer. Chem. Soc. 81, 6084.

LERNER, A.B., CASE, J.D., TAKAHASHI, Y., LEE, T.H. and MORI, W. (1958): Isolation of melatonin, the pineal gland factor that lightens melanocytes. J. Amer. Chem. Soc. 80, 2587.

LEWY, A.J., and MARKEY, S.P. (1978): Analysis of melatonin in human plasma by gas chromatography negative chemical ionization mass spectrometery. Science, 201, 741-743.

LEWY, A.J., WEHR, T.A., NURNBERGER, J.I., BECKER, L.E. ROSENTHAL, N.E., MARKEY, S.P., GOODWIN, K.K., NEWSOME, D.A. (1981): Suppression of human melatonin secretion by light: normal and abnormal responses. Abstr. 609. 63rd Annual Meeting of the Endocrine Society, Cincinnati, June, 1981.

LEWY, A.J., WEHR, T.A., GOODWIN, F.K., NEWSOME, D.A. and MARKEY, S.P. (1980): Light suppresses melatonin secretion in humans. Science, 210, 1267-1269.

LINCOLN, G.A. (1978): The photoperiodic control of seasonal breeding in rams. Comparative Endocrinology. pp. 149-152. Eds: P.J. Gaillard and H.H. Boer, Elsevier/North-Holland Biomedical Press, Amsterdam.

LINCOLN, G.A. (1979a): Photoperiodic control of seasonal breeding in the ram: participation of the cranial sympathetic nervous system. J. Endocrinol. 82, 135-147.

LINCOLN, G.A. (1979b): Light-induced rhythms of prolactin secretion in the ram and the effect of cranial sympathectomy. Acta endocrinol. 91, 421-427.

LINCOLN, G.A. (1980): Photoperiodic control of seasonal breeding in rams - the significance of short day refractoriness. In: Endocrinology, 1980. Eds: I.A. Cumming, J.W. Funder and F.A.O. Mendelsohn, Australian Academy of Science, Canberra. 283-286.

LINCOLN, G.A. (1981): Seasonal aspects of testicular function. In: The Testis. Eds: H. Burger and D. de Kretser, Raven Press, New York. pp. 255-302.

LINCOLN, G.A. (1983): The Pineal Gland. In: Reproduction in Mammals - Book 3. Eds: C.R. Austin and R.V. Short, Cambridge University Press (in press).

LINCOLN, G.A. and ALMEIDA,O.F.X. (1982): Melatonin and the seasonal photoperiodic response in sheep. Reprod. Nutr. Develop. (in press).

LINCOLN, G.A., ALMEIDA, O.F.X., and ARENDT, J. (1981): Role of melatonin and circadian rhythms in seasonal reproduction in rams. J. Reprod. Fertil. Suppl. 30, 23-31.

LINCOLN, G.A., ALMEIDA, O.F.X., KLANDORF, H. and CUNNINGHAM, R.A. (1982): Hourly fluctuations in the blood levels of melatonin, prolactin, luteinizing hormone, follicle stimulating hormone, testosterone, triiodothyronine, thyroxine and cortisol in rams under artificial photoperiods, and the effects of cranial sympathectomy. J. Endocrinol. 92, 237-250.

LINCOLN, G.A. and DAVIDSON, W. (1977): The relationship between sexual and aggresive behaviour and pituitary and testicular activity during the seasonal sexual cycle of rams, and the influence of photoperiod. J. Reprod. Fertil. 49, 267-276.

LINCOLN, G.A., KLANDORF, H. and ANDERSON, N. (1980): Photoperiodic control of thyroid function and wool and horn growth in rams and the effect of cranial sympathectomy. Endocrinology, 107, 1543-1548.

LINCOLN, G.A., McNEILLY, A.S. and CAMERON, C.L. (1978): The effects of a sudden decrease or increase in day length on prolactin secretion in the ram. J. Reprod. Fertil. 52, 305-311.

LINCOLN, G.A., PEET, M.J. and CUNNINGHAM, R.A. (1977): Seasonal and circadian changes in the episodic release of follicle-stimulating hormone, luteinizing hormone and testosterone in rams exposed to artificial photoperiods. J. Endocrinol. 72, 337-349.

LINCOLN, G.A. and SHORT, R.V. (1980): Seasonal breeding: Nature's contraceptive. Rec. Prog. Horm. Res. 36, 1-52.

LOFTS, B. and MURTON, R.K. (1968): Photoperiodic and physiological adaptations regulating avian breeding cycles and their ecological significance. J. Zool.(London),155, 327-394.

LOSSEE, S.H. and TUREK, F.W. (1981): Melatonin treatment prevents the termination of the gonadal refractory condition normally observed in hamsters exposed to long days. In: Pineal Function. Eds: C.D. Matthews and R.F. Seamark. Elsevier/ North Holland Biomedical Press, Amsterdam. pp. 67-75.

LYNCH, H.J., OZAKI, Y. and WURTMAN, R.J. (1978): The measurement of melatonin in mammalian tissues and body fluids. J. Neural Transm. Suppl. 13, 251-264.

LYNCH, H.J., RIVEST, R.W., RONSHEIM, P.M. and WURTMAN, R.J. (1981): Light intensity and the control of melatonin secretion in rats. Neuroendocrinology, 33, 181-185.

LYNCH, H.J. and WURTMAN, R.J. (1979): Control of rhythms in the secretion of pineal hormones in humans and experimental animals. In: Biological Rhythms and their Central Mechanism. pp. 118-131. Eds: M. Suda, O. Hayaishi and H. Nakagawa. Elsevier/North Holland Biomedical Press, Amsterdam.

MARSHALL, F.H.A. (1937): On the change-over in the oestrous cycle in animals after transference across the equator, with further observations on the incidence of the breeding seasons and the factors controlling sexual periodicity. Proc. R. Soc. B122, 413-

MARTIN, J.E., and KLEIN, D.C. (1976): Melatonin inhibition of the neonatal pituitary response to luteinizing hormone-releasing factor. Science 191, 301-302.

MARTIN, J.E. and SATTLER, C. (1982): Selectivity of melatonin pituitary inhibition for luteinizing hormone-releasing hormone. Neuroendocrinology 34, 112-116.

McCANN, S. (1981): CNS control of the pituitary. In: Neuroendocrinology of Reproduction. Ed: N.T. Adler, Plenum Press, New York, pp.427-450.

McCORD, C.P. and ALLEN, F.P. (1917): Evidence associating pineal gland function with alterations in pigmentation. J. Exp. Zool. 39, 11-16.

McNEILLY, A.S. and ANDREWS, P. (1974): Purification and characterization of caprine prolactin. J. Endocrinol. 60, 359-364.

MENAKER, M., HUDSON, D.J. and TAKAHASHI, J.S. (1981): Neural and endocrine components of circadian clocks in birds. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett. Wright, Bristol, pp. 171-183.

MIENO, M., YAMASHITA, E.R., IIMORI, M. and YAMASHITA, K. (1978): An inhibitory effect of melatonin on the luteinizing hormone releasing activity of luteinizing hormone in immature male digs. J. Endocrinol. 78, 283-284.

MILCU, S.M., PAVEL, S. and NEACSU, C. (1963): Biological and chromatographic characterization of a polypeptide with pressor and oxytocic activities isolated from bovine pineal gland. Endocrinology, 72, 563-566.

MILNER, C. and GWYNNE, D. (1974): The Soay sheep and the food supply. In: Island Survivors: The Ecology of the Soay Sheep of St. Kilda. Eds: P.A. Jewell, C. Milner and J. Morton Boyd. The Athlone Press, London, pp. 273-325.

MINNEMAN, K.P. and WURTMAN, R.J. (1976): The pharmacology of the pineal gland. Annu. Rev. Pharmacol. Toxicol. 16, 33-51.

MOORE, R.Y. (1973): Rentiohypothalamic projections in mammals: a comparative study. Brain Res. 49, 403-409.

MOORE, R.Y. (1978): Central Neural Control of circadian rhythms. Ch. 7 In: Frontiers in Neuroendocrinology Vol. 5 Eds: W.F. Ganong and L. Martini. Raven Press, N.Y.

MOORE, R.Y. (1979): The retinhypothalamic tract, supra-chiasmatic hypothalamic nucleus and central neural mechanisms of circadian rhythm regulation. In: Biological Rhythms and their Central Mechanism. Eds: M. Suda, O. Hayaishi and H. Nagegawa. Elsevier/North-Holland Biomedical Press, Tokyo. pp. 343-354.

MOORE, R.Y. and KLEIN, D.C. (1974): Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity. Brain Res. 71, 17-33.

MORIN, L.P., FITZGERALD, K.M., RUSAK, B. and ZUCKER, I. (1977): Circadian organization and neural mediation of hamster reproductive rhythms. Psychoneuroendocrinology 2, 73-98.

MORTIMER, D. and LINCOLN, G.A. (1982): Ultrastructural study of the regressed and reactivated testes from Soay rams. J. Reprod. Fert. 64, 437-442.

NANDA, K.R. and HAMNER, K.C. (1958): Studies on the nature of the endogenous rhythm affecting photoperiodic response of Biloxi soya bean. Bot. Gas. (Chicago) 120, 14-25.

NEWMAN TAYLOR, A. and WILSON, R.W. (1970): Electrophysiological evidence for the action of light on the pineal gland in the rat. Experientia, 26, 267-269.

NIESCHLAG, E. (1975): Immunization with hormones in reproduction research. North Holland Publishing Co, Amsterdam.

NILES, L.P., BROWN, G.M. and GROTA, L.J. (1977): Antigonadal effects of blinding in the presence of antibody against circulating melatonin. Life Sci. 21, 613-618.

NILES, L.P., BROWN, G.M., PANG, S.F. and GROTA, L.J. (1979): Pineal melatonin synthesis following neutralization of circulating N-acetylindolealkylamines. Intern. J. Pharmacol. 1, 213-217.

NISHINO, H., KOIZUMI, K. and BROOKS, C. McC., (1976): The role of the suprachiasmatic nuclei of the hypothalamus in the production of circadian rhythm. Brain Res. 112, 45-59.

NORDBLOM, G.D., WEBB, R. COUNSELL, R.E. and ENGLAND, B.G. (1981): A chemical approach to solving bridging phenomena in steroid radioimmunoassays. Steroids 38, 161-173.

OKSCHE, A. (1965): Survey of the development and comparative morphology of the pineal organ. Prog. Brain Res. 10, 4-29.

OKSCHE, A. (1971): Sensory and glandular elements of the pineal gland. In: The Pineal Gland. Eds: G.E.W. Wolstenholme and J. Knight. Churchill Livingstone, Edinburgh, pp. 127-146.

ORTS, R.J., BRUOT, B.C. and SARTIN, J.L. (1980): Inhibitory properties of a bovine pineal tripeptide,threonylseryl-lysine, on serum follicle-stimulating hormone. Neuronendocrinology, 31, 92-95.

OZAKI, Y., LYNCH, H.J. and WURTMAN, R.J. (1976): Melatonin in rat pineal plasma and urine: 24 hour rhythmicity and effect of chlopromazine. Endocrinology 98, 1418-1424.

PANKE, E.S., ROLLAG, M.D. and REITER, R.J. (1979): Pineal melatonin concentrations in the Syrian hamster. Endocrinology, 104, 194-197.

PANKE, E.S., ROLLAG, M.D. and REITER, R.J. (1980): Effects of photoperiod on hamster pineal melatonin concentrations. Comp. Biochem. Physiol., 66A, 691-693.

PAVEL, S., (1973): Arginine vasotocin release into cerebral spinal fluid of cats induced by melatonin. Nature, 246, 183-184.

PAVEL, S. (1978): Arginine vasotocin as a pineal hormone. J. Neural. Transm. Suppl. 13, 135-155.

PAVEL, S., LUCA, N., CALB, M. and GOLDSTEIN, R. (1979): Inhibition of release of luteinizing hormone in the male rat by extremely small amounts of arginine vasotocin: further evidence for the involvement of 5-hydroxytryptamine containing neurones in the mechanism of action of arginine vasotocin. Endocrinology, 104, 517-523.

PELLETIER, J. (1973): Evidence for photoperiodic control of prolactin release in rams. J. Reprod. Fert. 35, 143-147.

PELLETIER, J. and ORTAVANT, R. (1975): Photoperiodic control of LH release in the ram. I. Influence of increasing and decreasing light photoperiods. Acta Endocrinol (Copenh.) 78, 435-441.

PENGELLEY, E.T. (1974): Circannual Clocks. Academic Press, N.Y.

PENGELLEY, E.T. and ASMUNDSON, S.J. (1981): Annual biological clocks. Sci. Amer. 224, 72-79.

PERLOW, M.J., REPPERT, S.M., BOYAR, R.M. and KLEIN, D.C. (1981): Daily rhythms in cortisol and melatonin in primate cerebrospinal fluid. Neurendocrinology, 32, 193-196.

PIEKUT, D.T., and KNIGGE, K.M. (1981): Immunocytochemical analysis of the rat pineal gland using antisera generated against luteinizing hormone-releasing hormone (LHRH). J. Histochem. Cytochem. 29, 616-622.

PITTENDRIGH, C.S. and MINIS, D.H. (1964): The entrainment of circadian oscillations by light and their roles as photo-periodic clocks. Amer. Nat. 98, 261-294.

PRATT, J.J. (1978): Steroid immunoassay in clinical chemistry. Clin. Chem. 24, 1869-1890.

QUAY, W.B. (1956): Volumetric and cytologic variation in the pineal body of Peromyscus leucopus (Rodentia)with respect of sex, captivity and day length. J. Morph. 98, 471-495.

QUAY, W.B. (1973): Retrograde perfusions of the pineal region and the question of pineal vascular routes to brain and choroid plexuses. Am. J. Anat. 137, 387-402.

QUAY, W.B. (1976): Pineal Biochemistry. C.C. Thomas, Springfield, Illinois.

RADFORD, H.M. (1961): Photoperiodism and sexual activity in Merino ewes II. The effect of equinoctial light on sexual activity. Austr. J. Agric. Res. 12, 147-153.

RALPH, C.L. (1975): The pineal gland and geographical distribution of animals. Intern. J. of Biometer. 19, 289-303.

RALPH, C.L. (1981): Melatonin production by extra-pineal tissues. In: Melatonin: Current Status and Perspectives. Eds: N. Birau and W. Schloot. Pergamon Press, Oxford. pp. 35-46.

RALPH, C.L., BINKLEY, S., MACBRIDE, S.E. and KLEIN, D.C. (1975): Regulation of pineal rhythms in chickens: effects of blinding, constant light, constant dark, and superior cervical ganglionectomy. Endocrinology 97, 1373-1378.

RAVAULT, J.P. (1976): Prolactin in the ram: seasonal variation in the concentration of blood plasma from birth to three years. Acta Endocrinol. (Copenh). 78, 435-441.

RAVAULT, J.P. and ORTAVANT, R. (1977): Light control of prolactin secretion in sheep. Evidence for a photoinducible phase during a diurnal rhythm. Annales de Biologique Animale, Biochimie, Biophysique, 17, 459-473.

REITER, R.J. (1972): Evidence for refractoriness of the pituitary-gonadal axis to the pineal gland and its possible implication in annual reproductive cycles. Anat. Rec. 173, 365-371.

REITER, R.J. (1975): Exogenous and endogenous control of the annual reproductive cycle in the male golden hamster: participation of the pineal gland. J. Exper. Zool. 191, 111-119.

REITER, R.J. (1980): The pineal and its hormones in the control of reproduction in mammals. Endocrine Rev. 1, 109-131.

REITER, R.J., BLASK, D.E., JOHNSON, L.Y., RUDEEN, P.K., VAUGHAN, M.K. and WARING, P.J. (1976): Melatonin inhibition of reproduction in the male hamster: its dependency on time of day of administration and on the intact and sympathetically innervated pineal gland. Neuroendocrinology, 22, 107-116.

REITER, R.J., PETTERBORG, L.T. and PHILO, R.C. (1979): Refractoriness to the antigonadotrophic effects of melatonin in male hamsters and its interruption by exposure of the animals to long daily photoperiods. Life Sci. 25, 1571-1576.

REITER, R.J., SORRENTINO, S. and HOFFMAN, R.A. (1980): Early photoperiodic conditions and pineal antigonadal function in male hamsters. Science 185, 1169-1171.

REITER, R.J., VAUGHAN, M.K. and BLASK, D.E. (1975): Possible role of the cerebrospinal fluid in the transport of pineal hormones in mammals. In: Brain-Endocrine Interaction II: the ventriculer system. Eds: K.M. Knigge and D.E. Scott, Karger, Basal, pp. 337-354.

REITER, R.J., VAUGHAN, M.K., BLASK, D.E. and JOHNSON, L.Y. (1974): Melatonin: its inhibition of pineal anti-gonadotrophic activity in male hamsters. Science, 185, 1169-1171.

REPPERT, S.M., PERLOW, M.J., TAMARKIN, L. and KLEIN, D.C. (1979): A diurnal melatonin rhythm in primate cerebrospinal fluid. Endocrinology, 104, 295-301.

RISSMAN, E.F. (1980): Prepubertal sensitivity to melatonin in male hamsters. Biol. Reprod. 22, 277-280.

ROLLAG, M.D., MORGAN, R.J. and NISWENDER, G.D. (1977): Route of melatonin secretion in sheep. Biomed. Sci. Instrum. 13, 111-117.

ROLLAG, M.D., MORGAN, R.J. and NISWENDER, G.D. (1978): Route of melatonin secretion in sheep. Endocrincology, 102, 1-8.

ROLLAG, M.D. and NISWENDER, G.D. (1976): Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimes. Endocrinology. 98, 482-489.

ROLLAG, M.D., O'CALLAGHAN, P.L. and NISWENDER, G.D. (1978): Serum melatonin concentrations during different stages of the annual reproductive cycle in ewes. Biol. Reprod. 18, 279-285.

ROLLAG, M.D., PANKE, E.S. and REITER, R.J. (1980a): Pineal melatonin content in male hamsters throughout the seasonal reproductive cycle. Proc. Soc. Explt. Biol. Med. 165, 330-334.

ROLLAG, M.D., PANKE, E.S., TRAKULRUNGSI, W., TRAKULRUNGSI, C., and REITER, R.J. (1980b): Quantification of daily melatonin synthesis in the hamster pineal gland. Endocrinology, 106, 231-236.

ROLLESTON, H.D. (1936): The Endocrine Organs in Health and Disease (with an historical review). Oxford University Press, London, pp. 452-470.

ROTH, J.J., GERN, W.A., ROTH, E.C., RALPH, C.L. and JACOBSON, E. (1980): Non-pineal melatonin in the alligator (Alligator mississippiensis). Science, 210, 548-550.

ROWAN, W. (1925): Relation of light to bird migration and developmental changes. Nature, 115, 494-495.

RUSAK, B., and MORIN, L.P. (1976): Testicular responses to photoperiod are blocked by lesions of the suprachiasmatic nuclei in golden hamsters. Biol. Reprod. 15, 366-374.

RUSAK, B. (1980): Suprachiasmatic lesions prevent an antigonadal effect of melatonin. Biol. Reprod. 22, 148-154.

RUSAK, B. (1981): Mammals - general discussion. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follet and D.E. Follett, Wright, Bristol. pp. 219-222.

RUSAK, B. and ZUCKER, I. (1979): Neural regulation of circadian rhythms. Physiological Reviews, 59, 449-526.

RUST, C.C. and MEYER, R.K. (1969): Hair color, molt, and testis size in male short-tailed weasels treated with melatonin. Science, 165, 921-922.

SAKUMA, Y. and PFAFF, D.W. (1980): LH-RH in the mesencephalic central grey can potentiate lordosis reflex of female rats. Nature, 283, 566-567.

SAUNDERS, D.S. (1970): Circadian clocks in insect photoperiodism. Science, 168, 601-603.

SAUNDERS, D.S. (1977): An Introduction to Biological Rhythms. Blackie: Glasgow and London. 170p.

SCHÄFER, E.A. (1907): On the incidence of daylight as a determining factor in bird migration. Nature, 77, 159-163.

SEAMARK, R.F., KENNAWAY, D.J., MATTHEWS, C.D., FELLENBERG, A.J., PHILLIPOU, G., KOTARAS, P., McINTOSH, J.E.A., DUNSTAN, E., and OBST, J.M. (1981): The role of the pineal gland in seasonality. J. Reprod. Fert. Suppl. 30, 15-21.

SEMM, P.,SCHNEIDER, T. and VOLLRATH, L. (1981): Morphological and electrophysiological evidence for habenular influence on the guinea-pig pineal gland. J. Neural Transm. 50, 247-266.

SEMM, P. and VOLLRATH, L. (1979): Electrophysiology of the guinea-pig pineal organ: sympathetic influence and different reactions to light and darkness. Prog. Brain Res. 52, 107-110.

SHARPE, R.M. (1979): Regulation of LH-Receptors and steroidogenic responsiveness of the rat testis. Ph.D Thesis. Council for National Academic Awards (UK). 242p.

SHORT, R.V. (1973): Preface: a general discussion of the effects of the environment on reproduction. J. Reprod. Fertil. Suppl. 19, ix-xiii.

SIEGEL, S. (1956): Non-parametric Statistics, McGraw-Hill Book, Inc. New York. 312.

SINGER, C. (1956): Galen on Anatomical Procedures. Oxford University Press, London.

SHARP, D.C., GRUBAUGH, W., BERGLUND, L.A., SEAMANS, K.W. and ASQUITH, R.L. (1980). Biol. Reprod. Suppl. 1. Vol. 22.

SKELLY, D.S., BROWN, L.P. and BESCH, P.K. (1973): Radioimmunoassay. Clin. Chem. 19, 146-186.

SMITH, I., FRANCIS, P., LEONE, R.M. and MULLEN, P.E. (1980): Identification of O-acetyl-5-methotytryptophol in the pineal gland by gas chromatography and mass spectrometery. Biochem. J. 185, 537-540.

SMITH, J.A. (1981): The pharmacology of melatonin. In: Melatonin: Current Status and Perspectives. Eds: N. Birau and W. Schloot. Pergamon Press, Oxford, pp.113-118.

SOLLBERGER, A. (1965): Biological Rhythm Research. Elsevier Publishing Co. Amsterdam, 461p.

STEPHAN, F.K. and ZUCKER, I. (1972): Circadian rhythms in drinking behaviour and locomotor activity are eliminated by suprachiasmatic lesions. Proc. Natl. Acad. Sci. USA, 69, 1583-1586.

STETSON, M.H., ELLIOTT, J.A. and MENAKER, M. (1975): Photoperiodic regulation of hamster testis: circadian sensititvity to effect of light. Biol. Reprod. 13, 329-339.

STETSON, M.H., MATT, K.S. and WATSON-WHITMYRE, M. (1976): Photoperiodism and reproduction in golden hamsters: circadian organization and the termination of photo-refractoriness. Biol. Reprod. 14, 531-537.

STETSON, M.H., and TATE-OSTROFF, B. (1981): Hormonal regulation of the annual reproductive cycle of golden hamsters. Gen. Comp. Endocrinol. 45, 329-344.

STETSON, M.H. and WATSON-WHITMYRE, M. (1976): Nucleus suprachiasmaticus: the biological clock of the hamster? Science, 191, 197-199.

STETSON, M.H., WATSON-WHITMYRE, M. and MATT, K.S. (1977): Termination of photorefractoriness in golden hamsters - photoperiodic requirements. J. Expl. Zool. 202, 81-88.

STOREY, C.R. and NICHOLLS, T.J. (1978): Failure of exogenous melatonin to influence the maintenance or dissipation of photo-refractoriness in the Canary, Serinus canarius. Gen. Comp. Endocrinol. 34, 468-470.

SYMONS, A.M. and ARENDT, J. (1982): Lack of effect of melatonin on the pituitary response to LH-RH in the ewe. J. Reprod. Fertil. 64, 103-106.

SZMUSZKOVICZ, J., ANTHONY, W.C. and HEINZELMAN, R.V. (1960): Synthesis of N-acetyl-5-methoxytryptamine. J. Organ. Chem. 25, 857.

TAKAHASHI, J.S., HAMM, H. and MENAKER, M. (1980): Circadian rhythms of melatonin release from individual superfused chicken pineal glands in vitro. Proc. Natl. Acad. Sci. USA, 77, 2319-2322.

TAMARKIN, L., HOLLISTER, C.W., LEFEBVRE, N.G. and GOLDMAN, B.D. (1977a). Melatonin induction of gonadal quiescence in the pinealectomized Syrian hamsters. Science, 198, 935-937.

TAMARKIN, L., LEFEBVRE, N.G., HOLLISTER, C.W. and GOLDMAN, B.D. (1977b): Effect of melatonin administered during the night on reproductive function in the Syrian hamster. Endocrinology, 101, 631-634.

TAMARKIN, L. REPPERT, S.M. and KLEIN, D.C. (1979): Regulation of pineal melatonin in the Syrian hamster. Endocrinology, 104, 385-389.

TAMARKIN, L., REPPERT, S.M., KLEIN, D.C., PRATT, B., and GOLDMAN, B.D. (1980): Studies on the daily pattern of pineal melatonin in the Syrian hamster. Endocrinology, 107, 1525-1529.

TAMARKIN, L., WESTROM, W., HAMILL, A. and GOLDMAN, B. (1976): Effect of melatonin on the reproductive systems of male and female Syrian hamsters: a diurnal rhythm in sensitivity to melatonin. Endocrinology, 99, 1534-1541.

THIBAULT, C., COUROT, M., MARTINET, L., MAULEON, P. du MESNIL du BUISSON, F., ORTAVANT, R., PELLETIER, J. and SIGNORET, J.P. (1966): Regulation of breeding seasons and estrous cycles by light and external stimuli in some mammals. J. Anim. Sci. Suppl. 25, 119-142.

THIMONIER, J. (1981): Control of seasonal reproduction in sheep and goats by light and hormones. J. Reprod. Fert. Suppl. 30, 33-45.

THORPE, P. and HERBERT, J. (1976): Studies on the duration of the breeding seasons and photorefractoriness in female ferrets pinealectomized or treated with melatonin. J. Endocrinol. 70, 255-262.

THWAITES, C.J. (1965): Photoperiodic control of breeding activity in the southdown ewe with particular reference to the effects of an equatorial light regime. J. Agric. Sci. Cambridge, 65, 57-64.

TIETJEN, G.L. and MOORE, R.H. (1972): Some Grubbs-Type Statistics for the detection of outliers. Technometrics, 14, 583-597.

TRENTINI, G.P., MESS, B., de GAETANI, C.F. and RUZSAS, C. (1979): Pineal-brain relationship. Prog. Brain Res. 52, 341-365.

TUREK, F.W. (1972): Circadian involvement in termination of the refractory period in two sparrows. Science, 178, 1112-1113.

TUREK, F.W. (1977): Antigonadal effect of melatonin in pinealectomized and intact male hamsters. Proc. Soc. Exptl. Biol. Med. 155, 31-34.

TUREK, F.W. (1979a): Effect of melatonin on photoc-independent and photoc-dependent testicular growth in juvenile and adult male golden hamsters. Biol. Reprod. 20, 1119-1122.

TUREK, F.W. (1979b): Role of the pineal gland in photo period-induced changes in hypothalamic-pituitary sensitivity to testosterone feedback in castrated male hamsters. Endocrinology, 104, 636-640.

TUREK, F.W. and CAMPBELL, C.S. (1979): Photoperiodic regulation of neuroendocrine-gonadal activity. Biol. Reprod. 20, 32-50.

TUREK, F.W., DESJARDINS, C. and MENAKER, M. (1975): Melatonin: Antigonadal and progonadal effects in male golden hamsters. Science, 190, 280-282.

TUREK, F.W., DESJARDINS, C. and MENAKER, M. (1976a): Differential effects of melatonin on the testes of photo-periodic and non-photoperiodic rodents. Biol. Reprod. 15, 94-97.

TUREK, F.W., DESJARDINS, C. and MENAKER, M. (1976b): Melatonin-induced inhibition of testicular function in adult golden hamsters. Proc. Soc. Exp. Biol. Med. 151, 502-506.

TUREK, F.W., ELLIOTT, J.A., ALVIS, J.D. and MENAKER, M. (1975): Effect of prolonged exposure to non-stimulatory photoperiods on the activity of the neuroendocrine-testicular axis of golden hamsters. Biol. Reprod. 13, 475-481.

TUREK, F.W. and ELLIS, G.B. (1980): Role of the pineal gland in seasonal changes in neuroendocrine-testicular function. In: Testicular Development, Structure and Function. Eds: A. Steinberger and E.E. Steinberger, Raven Press, N.Y. pp. 389-393.

TUREK, F.W. and LOSEE, S.H. (1978): Melatonin-indiced testicular growth in golden hamsters maintained on short days. Biol. Reprod. 18, 299-305.

TUREK, F.W. and LOSEE, S.H. (1979): Photoperiodic inhibition of the reproductive system: a prerequisite for the induction of the refractory period in hamsters. Biol. Reprod. 20, 611-616.

TUREK, F.W., McMILLAN, J.P. and MENAKER, M. (1976): Melatonin: effects on the circadian locomotor rhythm of sparrows. Science, 194, 1441-1443.

TUREK, F.W. and PAPPAS, P. (1980): Inhibition of short-day induced testicular regression in the hamster by daily melatonin injections. Experientia, 36, 1426-1427.

TUREK, F.W. and WOLFSON, A. (1978): Lack of an effect of melatonin treatment via silastic capsules on photic-induced gonadal growth and the photorefractory condition in white-throated sparrows. Gen. Comp. Endocrinol. 34, 471-474.

UECK, M. (1979): Innervation of the vertebrate pineal. Prog. Brain Res. 52, 45-87.

UNDERWOOD, H. (1981): Circadian clocks in lizards: Photo-reception physiology and photoperiodic time measurement. In: Biological Clocks in Seasonal Reproductive Cycles. Eds: B.K. Follett and D.E. Follett. Wright, Bristol. pp. 137-152.

VAITUKAITIS, J., ROBBINS, J.B., NIESCHLAG, E. and ROSS, G.T. (1971): A method for producing specific antisera with small doses of immunogen. J. Clin. Endocrinol. 33, 988-991.

VAUGHAN, G.M., McDONALD, S.D., JORDAN, R.M., ALLEN, J.P., BELL, R. and STEVENS, E.A. (1979): Melatonin, pituitary function and stress in humans. Psychoneuroendocrinology, 4, 351-361.

VOLLRATH, L. (1979): Comparative morphology of the vertebrate pineal complex. Prog. Brain Res. 52, 25-37.

VRIEND, J., HINKLE, P.M., and KNIGGE, K.M. (1980): Evidence for a thyrotropin-releasing hormone inhibitor in the pineal gland. Endocrinology, 107, 1791-1797.

de WIED, D. (1979): Neuropeptides and memory. Acta. Endocrinol. (Copenh) Suppl. 225, 416-418.

WILKINSON, M., ARENDT, J., BRADTKE, J. and de ZIEGLER, D. (1977): Determination of a dark-induced increase of pineal N-acetyltransferase activity and simultaneous radioimmunoassay of melatonin in pineal, serum, and pituitary tissue of the male rat. J. Endocrinol. 72, 243-244.

WINER, B.J. (1971): Statistical principles in experimental design. 2nd Edin. McGraw-Hill, Kogakushu Ltd., Tokyo.

WURTMAN, R.J., AXELROD, J. and KELLY, D.E. (1968): The Pineal Gland. Academic Press, New York. 199p.

WURTMAN, R.J., ROSE, C.M., CHOU, C. and LARIN, F. (1968): Daily rhythms in the concentrations of various animo acids in human plasma. New Engl. J. Med. 279, 171-175.

WURTMAN, R.J., SHEIN, H.M., AXELROD, J. and LARIN, F. (1969): Incorporation of ^{14}C-trytophan into ^{14}C-protein by cultured rat pineals: stimulation by L-norepinephrine. Proc. Natl. Acad. Sci. USA, 62, 749-755.

YALOW, R.S. and BERSON, S.A. (1968): Radioisotopes in Medicine: In vitro studies. U.S. Atomic Energy Commission, Oak Rodge, Tennessee, p7.

YAMASHITA, K., MIENO, M., SHIMIZU, T. and YAMASHITA, E. (1978): Inhibition by melatonin of the pituitary response to luteinizing hormone releasing hormone in vivo. J. Endocrinol. 76, 487-491.

YATES, C.A., and HERBERT, J. (1976): Differential circadian rhythms in pineal and hypothalamic 5-HT induced by artificial photoperiods or melatonin. Nature, 262 , 219-220.

YEATES, N.T.M. (1949): The breeding season of the sheep with particular reference to its modification by artificial means using light. J. Agric. Sci. 39, 1-43.

YOUNGBLOOD, W.W., HUNN, J. and KIZER, J.S. (1979): TRH-like immunoreactivity in rat pancreas and eye, bovine and sheep pineals, and human placenta: non-identity with synthetic pyroglu-his-proNH$_2$ (TRH). Brain Res. 163, 101.

ZATZ, M. (1979): A neuropharmacological approach to the circadian oscillator regulating rat pineal serotonin N-acetyltransferase activity. In: Biological Rhythms and their Central Mechanism. Eds: M. Suda, O. Hayaishi and H. Nakagawa. Elsevier/North Holland Biomedical Press, Tokyo, p.149-158.

ZIMMERMAN, N.H. and MEKAKER, M. (1979): The pineal gland: A pacemaker within the circadian system of the house sparrow. Proc. Natl. Acad. Sci. USA. 76, 999-1003.

ZUCKER, I., JOHNSTON, P.G. and FROST, D. (1980): Comparative, physiological and biochronometric analyses of rodent seasonal reproductive cycles. Prog. Reprod. Biol. 5, 102-133.